PATHOGENICITY MECHANISMS OF *CAMPYLOBACTER JEJUNI* AND *CAMPYLOBACTER FETUS*: CHARACTERIZATION OF PATHOGENICITY FACTORS AND SIGNALING IN HOST CELL INVASION

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„Das Schönste, was wir erleben können,
ist das Geheimnisvolle.
Es ist das Grundgefühl,
das an der Wiege von wahrer Kunst
und Wissenschaft steht.
Wer es nicht kennt
und sich nicht mehr wundern,
nicht mehr staunen kann,
der ist sozusagen tot
und sein Auge erloschen.“

Albert Einstein

„The most beautiful thing we can experience,
is the mysterious.
It is the source of all true art and all science.
He to whom this emotion is a stranger,
who can no longer pause to wonder
and stand rapt in awe,
is as good as dead: his eyes are closed.“

Albert Einstein

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Abbreviations

A, C, G, T	Adenine, Cytosine, Guanine, Thymine
AIDS	Acquired Immunodeficiency Syndrome
Amp^R, Kan^R, Neo^R	Resistance to ampicillin, kanamycin, neomycin
Arp2/3 complex	Actin-related protein 2/3 complex
ATP	Adenosine-5'-tri-phosphate
BCA	Bicinchoninic acid
BHI	Brain Heart Infusion
bp	Base pair
BSA	Bovine serum albumin
CA	Constitutively-active
CadF	*Campylobacter* adhesion to fibronectin
Cdc42	Cell division cycle 42
cDNA	Complementary deoxyribo nucleic acid
CDT	Cytolethal distending toxin
CFU	Colony forming unit
Cia	*Campylobacter* invasion antigens
CLSM	Confocal Laser Scanning Microscopy
CMV promoter	Human cytomegalovirus promoter
CNF-1, CNF-Y	Cytotoxic necrotizing factor 1, Y
CPS	Capsular polysaccharide
CRIB	Cdc42/Rac1 Interactive Binding
DCs	Dendritic cells
D-MEM	Dulbecco's-Modified Eagle Medium
DN	Dominant-negative
DOCK180	Dedicator of cytokinesis 180
dsRNA, mRNA, rRNA, siRNA	Double stranded, messenger, ribosomal, small interfering ribonucleic acid
e.g.	For example (*exempli gratia*)
ECM	Extracellular matrix
EDTA	Ethylene diamine tetracetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EPEC	Enteropathogenic *Escherichia coli*
et al.,	And others (*et alii*)
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FESEM	Field Emission Scanning Electron Microscopy
FITC	Fluorescein isothiocyanate
Fn	Fibronectin
GAP	GTPase-activating protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GBS	Guillain-Barré syndrome
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
Grb2	Growth factor receptor-bound protein 2
GROα, GROγ	Growth-related oncogene α, γ
GST	Glutathione S-transferase
GDP, GTP	Guanosine 5' di-, tri-phosphate
GTPase	Guanosine triphosphatase
HIV	Human immunodeficiency virus
IF	Immunofluorescence
IgG	Immunoglobulin Class G
IL-8	Interleukin 8
INT-407	Intestinal epithelial cells
IP	Immunoprecipitation
IPTG	Isopropyl β-D-thiogalactopyranoside
JlpA	*jejuni* lipoprotein A
kDa	Kilodalton
LB	Luria-Bertani

LOS	Lipooligosaccharide
MALDI-MS	Matrix-assisted laser desorption/ionization-mass spectrometry
MAP	Mitogen-activated protein
MCP-1	Monocyte chemoatractant protein 1
MCS	Multiple cloning site
MEM	Eagle's Minimum Essential Medium
MH	Mueller-Hinton
MIP-1α, MIP-3α	Macrophage inflammatory protein 1α, 3α
MOI	Multiplicity of infection
MOMP	Major outer membrane protein
NF-κB	Nuclear factor κB
NOD	Nucleotide-binding oligomerization domain
OD_{600}	Optical density at λ=600 nm
ori	Origin of replication
P130 Cas	Crk-associated substrate
PAF	Population attributable fraction
PAK	p21 activated kinase
PBD	p21 Binding Domain
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
PEB1	Periplasmic binding protein
PFA	Paraformaldehyde
Pgl	Protein glycosylation
PI3 kinase	Phosphatidylinositol 3 kinase
PolyA	Poly-adenylate
PTK	Protein-tyrosine kinase
PY	Phospho-tyrosine
Rac1	Ras-related C3 botulinum toxin substrate 1
RhoA	Ras homolog gene family, member A
RISC	RNA-induced silencing complex
RNAi	RNA interference
Rpm	Rounds per minute
RPMI	Roswell Park Memorial Institute Media
RT	Room temperature
RTK	Receptor tyrosine kinase
SapA	Surface array protein
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SH2, SH3	Src homology 2, 3
S-layer	Surface layer
SLPs	S-layer proteins
TBS	Tris buffer saline
TCA cycle	Tricarboxylic acid cycle
TER	Transepithelial electrical resistance
Tiam1	T-cell lymphoma invasion and metastasis 1
TLR	Toll-like receptor
Trio	Triple functional domain
TRITC	Tetramethylrhodamine isothiocyanate
TSS1, T3SS, T4SS	Type I, III, IV secretion system
U	Unit of enzyme activity (μmol min^{-1} mg $protein^{-1}$)
v/v	Volume per volume
w/v	Weight per volume
wt	Wild-type
x *g*	x-times acceleration due to gravity (9,81m/s^2)
YT	Yeast tryptone
α-PIX	PAK interacting exchange factor
γIP-10	Gamma interferon-inducible protein 10

1. Abstract

Campylobacter jejuni is the leading bacterial cause of food-borne illness worldwide and a major cause of Guillain-Barré paralysis. Invasion of host target cells has been reported as one of the primary reasons of tissue damage caused by this pathogen but molecular mechanisms are widely unclear. In the present study, I characterized the fibronectin-binding protein CadF as important pathogenicity factor expressed in all tested *C. jejuni* and *C. coli* strains. CadF is involved not only in adhesion but also required for maximal host cell invasion of *Campylobacter.* Additionally, the difference in molecular size and nucleotide sequence between CadF of *C. jejuni* and *C. coli,* described here, may potentially be applicable to discriminate these species in food and clinical specimens. Furthermore, detailed understanding of the signaling events induced by *C. jejuni* infection is presented in this study. It is shown that *C. jejuni* triggers membrane ruffling in the eukaryotic cell followed by invasion in a very specific manner first with its tip followed by the flagellar end. To pinpoint important signaling events involved in the *C. jejuni* invasion process, the role of small Rho family GTPases was examined for the first time. Using several molecular biological tools including specific GTPase-modifying toxins, inhibitors, siRNA and GTPase expression constructs it is shown that Rac1 and Cdc42, but not RhoA, are involved in *C. jejuni* invasion. In agreement with these observations, it was found that internalization of *C. jejuni* is accompanied by a time-dependent activation of both Rac1 and Cdc42. Furthermore, with use of ß1- and FAK-knockout cells, different expression constructs, siRNA and inhibitors it is shown that the integrins, EGFR, PDGFR, FAK, DOCK180, Vav-2, α-PIX and Tiam1 are critically involved in mediating *C. jejuni* invasion-promoting signals. It is proposed that activated integrins and PDGFR/EGFR interact during *C. jejuni* infection and trigger formation of various signaling complexes including FAK, DOCK180, Vav-2, α-PIX and Tiam1 leading to the activation of Rac1 and Cdc42 and stimulation of common downstream signaling pathways. This in turn causes actin rearrangements and efficient *C. jejuni* uptake. Moreover, evidence is presented that activation of Rac1 and Cdc42 involves the CadF protein and the flagellar apparatus. Thus, CadF appears to be a bi-functional protein enabling bacterial binding to host cells as well as stimulating integrin clustering, which subsequently can activate downstream factors triggering GTPase signaling in infected host cells. Collectively, results of this study suggest that *C. jejuni* invade host target cells by a unique mechanism and the activation of the integrins, FAK, Rac1 and Cdc42, but not RhoA plays a central role in this entry process.

Finally, the role of the surface array protein SapA and its phosphorylation in infection with *Campylobacter fetus* is here established. With use of SapA cloning, purification and *in vitro* tests as well as examination of SapA-non expressing strains, it is shown that Src-like PTKs mediate SapA phosphorylation and indicated that phosphorylated SapA plays significant role during *C. fetus* infection.

2. Zusammenfassung

Campylobacter jejuni zählt zu den weltweit häufigsten lebensmittelbedingten Erregern der bakteriellen Enteritis und kann das Guillain-Barré Syndrom als eine mögliche Spätfolge verursachen. Die infektiöse Enteritis kann in Einzelfällen, zum Beispiel bei immunsupprimierten Menschen und Kleinkindern tödlich verlaufen. Desweiteren ist sie von hoher volkwirtschaftlicher Bedeutung. Die Invasion des Pathogens *C. jejuni* in die epithelialen Zellen wird als wichtigste Ursache für die Gewebeschädigung beschrieben, über die molekularen Mechanismen war zu Beginn der Dissertation jedoch noch wenig bekannt.

In der vorliegenden Arbeit, konnte zunächst das Fibronektin-bindende Protein CadF als ein wichtiger Pathogenitätsfaktor charakterisiert werden. CadF wird in allen bisher getesteten *C. jejuni* und *C. coli* Stämme exprimiert. Es ist nicht nur an der Adhäsion beteiligt, sondern auch für eine maximale Invasion von *Campylobacter* notwendig. Die beobachteten Unterschiede in Molekulargröße und Nukleotidensequenzen zwischen den CadF-Proteinen aus *C. jejuni* und *C. coli* könnten zur Etablierung eines neuen Assays für die Identifizierung und Diskriminierung von CadF-expremierenden *C. jejuni* und *C. coli* in Lebensmitteln sowie in klinischen Proben genutzt werden. Weiterhin konnten wichtige neue Erkenntnisse über die *C. jejuni*-induzierten Signalwege mit dieser Arbeit dargestellt werden. Es wurde gezeigt, dass die Bakterien die Kräuselung der Zellmembran verursachen und in die Zelle mit ihrer Spitze, folgend durch ein flagellares Ende eindringen. Um die durch *C. jejuni*-induzierten Signalwege während der Invasion im Detail zu untersuchen, wurde zunächst die Rolle der kleinen Rho-GTPasen, welche wichtige Schaltstellen für die Signaltransduktion zum Aktin-Zytoskelett sind, näher untersucht. Durch die Verwendung von spezifischen GTPase-modifizieriende-Toxinen, Inhibitoren, siRNA, dominant-negativen und konstitutiv-aktiven Rho-GTPase-Konstrukten, sowie spezifischen „Pull-Down"-Assays mit anschließender Western-Blot-Analyse, war es möglich Rac1 und Cdc42, aber nicht RhoA, als an der *C. jejuni* Invasion beteiligte GTPasen zu identifizieren. Übereinstimmend mit diesen Daten konnte gezeigt werden, dass eine Internalisierung von *C. jejuni* durch eine zeitabhängige Aktivierung von Rac1 und Cdc42 begleitet ist. Darüber hinaus, konnte unter Verwendung von ß1- und FAK-knockout Zellen, verschiedenen Expressions-Konstrukten, siRNA und Inhibitoren die Beteiligung von Integrine, EGFR, PDGFR, FAK, DOCK180, Vav-2, α-PIX und Tiam1 bei der *C. jejuni* Invasion dargestellt werden. Diese Daten münden in einem neuen Modell zur *C. jejuni* Invasion. Dabei interagieren die aktivierten Integrine und EGFR/PDGFR während *C. jejuni* Infektion um die Bildung von verschiedenen Signalkomplexen, einschließlich FAK, DOCK180, Vav-2, α-PIX und Tiam1, auszulösen was zu einer Aktivierung von Rac1 und Cdc42, Stimulierung von gemeinsamen Downstream-Signalwegen und zu Aktin-Zytoskelettalen Veränderungen führt. So ist eine effiziente Internalisierung von *C. jejuni* möglich. Weiterhin wurde gezeigt, dass an der Aktivierung von Rac1 und Cdc42

CadF und der flagellare Apparat involviert sind. CadF scheint somit ein bi-funktionales Protein zu sein, das zum einen an der Adhäsion beteiligt ist und zum anderem die Integrine stimuliert und dadurch die GTPasen aktiviert. Zusammengefasst, weisen die in dieser Arbeit dargestellten Ergebnisse darauf hin, dass *C. jejuni* durch einen einzigartigen Mechanismus in die Zielzellen eindringt, und die Aktivierung von Integrinen, FAK, der Rho GTPasen Rac1 und Cdc42, allerdings nicht RhoA, eine entscheidende Rolle bei der *C. jejuni* Invasion spielen.

Schließlich wurde im Rahmen dieser Arbeit die Rolle des Surface-array-Proteins SapA während der Infektion mit *Campylobacter fetus* dargestellt. Mittels SapA-Klonierung, sowie der Reinigung und zahlreichen *in vitro* Untersuchungen, aber auch durch die Verwendung von SapA-negativen Stämmen konnte gezeigt werden, dass die Src-Kinase die SapA-Phosphorylierung vermittelt. Die Ergebnisse weisen darauf hin, dass die Phosphorylierung von SapA eine wichtige Rolle während der *C. fetus* Infektion spielt.

3. Introduction

3.1. Epidemiology of *Campylobacter* species

After successful isolation from human stool in the 1970s, *Campylobacter jejuni* rapidly has become the most commonly recognized cause of bacterial gastroenteritis in humans. Fastidious culture requirements (Skirrow, 1977) and difficulties with early attempts at genetic modification have hampered progress in understanding this organism compared with other enteric pathogens such as *Escherichia coli* and *Salmonella* species. Currently, however, there is a renaissance of interest in *Campylobacter*. The first completed *C. jejuni* genome project (Parkhill *et al.,* 2000) and a recent emergence of antibiotic-resistant strains (Engberg *et al.,* 2001; Luo *et al.,* 2005) provided the new impetus for further research into this pathogen.

Campylobacter are gram-negative, spiral-shaped, (0.2 to 0.8 µm wide and 0.5 to 5.0 µm long), non-spore-forming rods, possessing a polar flagellum, which exhibits a high degree of motility. They do not metabolize carbohydrates and use intermediates of the tricarboxylic acid (TCA) cycle as a source of energy. They are microaerophilic, requiring 5 to 10% oxygen, 3 to 15% carbon dioxide and grow best at temperatures ranging from 37°C to 42°C. After exposure to unfavorable environmental conditions, such as an increased oxygen concentration, or during prolonged culture, the cells round up to “coccoid forms”. The role of theses putative dormant forms remains controversial (reviewed in Ketley, 1997; Kist & Bereswill, 2001; Konkel *et al.,* 2001; Bereswill & Kist, 2003; Crushell *et al.,* 2004). Until now genome sequences of seven *Campylobacter* strains are available including four *C. jejuni* isolates NCTC11168 (Parkhill *et al*., 2000), 81-176 (Hofreuter *et al*., 2006) and CG8486 (Poly *et al.,* 2007) of human origin and RM1221 (Fouts *et al*., 2005) from chicken carcasses, *C. coli* strain RM2228, *C. lari* RM2100 and *C. upsaliensis* RM3195 (Fouts *et al*., 2005). Ten additional strains of *Campylobacter* have been sequenced by the Institute for Genomic Research (D. Fouts unpublished, reported by Poly *et al.,* 2007). The relatively small genome of *C. jejuni* is a singular, circular chromosome, 1.59–1.77 Mbp in size, with an average G+C ratio of 30.3–30.6%. High gene content of 94–94.3% makes it one of the most dense bacterial genomes sequenced to date (Parkhill *et al*., 2000; Fouts *et al*., 2005; Hofreuter *et al.,* 2006).

Although *C. jejuni,* responsible for 80–85% of all human enteric *Campylobacter* infections, is the best-studied member of the genus, there exist a number of other *Campylobacter* species of relevance to human disease (Moore *et al.,* 2005). Among seventeen described species belonging to the *Campylobacter* genus (Vandamme, 2000; Korczak *et al.,* 2006), *C. jejuni*, *C. coli* and *C. fetus* are the most frequently associated with the human illness (Konkel *et al.,* 2001). The true incidence of infection by other species such

as *C. lari*, *C. upsaliensis*, and *C. hyointestinalis* is still widely unclear (ACMSF, 1993; Butzler, 2004) and probably underreported (Bourke *et al.,* 1998; Engberg *et al.,* 2000). *Campylobacter jejuni* and related species are the major cause of human bacterial gastroenteritis and may be responsible for as many as 400–500 million cases worldwide each year (Friedman *et al.,* 2000). Statistical data show that *Campylobacter* infections cause considerable use of medication and health service burden. In the USA, it has been estimated that the human *Campylobacter* illness cost up to $ 6.2 billion per year (Forsythe, 2000). Remarkably, in many studies in the United States and other industrialized countries, *Campylobacter* were found to cause diarrheal disease more than 2-7 times as frequently as *Salmonella* species, *Shigella* species or *Escherichia coli* O157:H7 (Allos, 2001; Tam, 2001). According to the Robert Koch Institute Statistical Report, the annual incidence of reported *Campylobacter* cases in Germany was 63/100,000 with a total of about 52,000 cases in 2006. This constituted 44 % of all reported food-borne infections (Fig. 1).

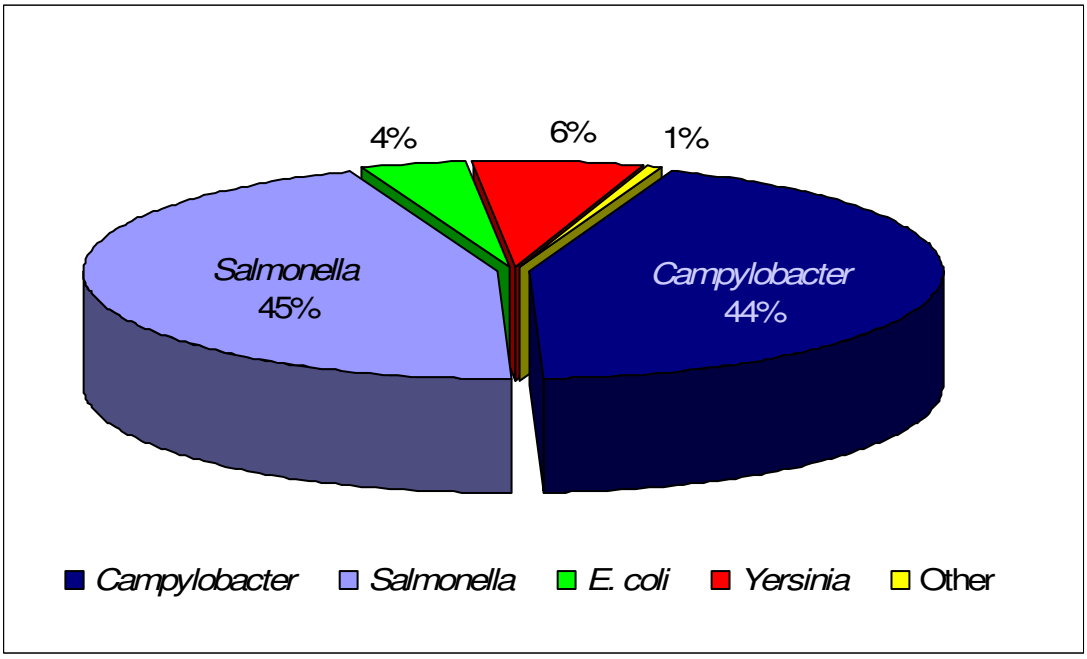


Fig. 1. The annual incidence of infections with pathogens transmitted commonly through food, based on German Disease Statistics Report for year 2006 published by Robert Koch Institute, Berlin, Germany.

Campylobacter jejuni and *C. coli* live predominantly as commensals in a wide range of wild and domestic birds and mammals, including poultry for food production, dairy cows as well as domestic pets. Their growth optimum 42°C is well adapted to the body temperature of birds. Swine are mainly colonized by *C. coli* (Kist & Bereswill, 2001; Newell, 2001). In contrast, *C. fetus* is a pathogen of cattle and sheep (Blaser, 1993). Whereas large-scale outbreaks of human campylobacteriosis are rare and usually linked to the consumption of contaminated water (Mentzing, 1981; Jones & Roworth, 1996; Koenraad *et al.,* 1997; Kuusi

et al., 2005; Schuster *et al.,* 2005) or raw milk (CDC, 1983; 2002; Korlath *et al*., 1985; Evans *et al.,* 1996; Frost *et al.,* 2002), sporadic cases are more common (Friedman *et al.,* 2004). *Campylobacter*-contaminated chickens' carcasses constitute the largest potential source of human sporadic infections (Adak *et al*., 1995; 2005). In a recent study aimed to determine the risk factors for *Campylobacter* infection in the USA, the largest population attributable fraction (PAF) of 24% was associated with the consumption of the chicken prepared in restaurants (Friedman *et al.,* 2004). Chickens are also an important source of human campylobacteriosis in developing countries (Coker *et al*., 2002). In these countries, where *Campylobacter* are hyperendemic owing to poor sanitation and close contact with animals, infection is very common in early childhood, with five to ten separate episodes occurring in the first two years of life (Taylor *et al.,* 1993; Lindblom *et al*., 1995; Coker *et al.,* 2002). Consequently, campylobacteriosis significantly contributes to malnutrition in infants, who represent the highest risk group (Butzler, 2004). In developed countries, *Campylobacter* affects all age groups, but infants and young adults have the highest reported rates of disease (Allos & Blaser, 1995). *Campylobacter* infections in industrialized countries typically result in acute inflammatory enteritis, whereas similar infections in developing countries tend to result in watery, non-inflammatory diarrhea. However, a spectrum of disease symptoms can be triggered by *Campylobacter*, regardless of geographical location (Taylor, 1992; Oberhelman & Taylor, 2000). It is not clear what mechanism underlies the different clinical presentations of infections in patients from developed and developing countries. It seems reasonable to presume that *Campylobacter* pathogenesis is largely influenced by the relative susceptibility of the host, but can also be affected by the relative virulence of the infecting strain. Probably both the bacterial and the host factors play a role for the diverse clinical manifestations.

3.2. Clinical manifestations

The most common illness associated with *C. jejuni* infection in humans is enteritis, the inflammation of small intestine. Frequently, a prodromal period lasting from a few hours to a few days and characterized by headache, back pain, myalgia and low fever precedes the acute phase of the disease, which often starts with severe abdominal cramps, followed by up to 20 watery evacuations per 24 hrs, or gross bloody diarrhea in a remarkable number of cases (Kist & Bereswill, 2001). Although *C. jejuni* enteritis is generally considered a benign self-limiting illness, there is a significant morbidity and mortality for humans, even in developed countries (Mead *et al.,* 1999). Individuals suffering from AIDS are at a greater risk of acquiring *C. jejuni* than the background population (Butzler, 2004). *Campylobacter jejuni* infections can be accompanied by bacteremia, septic arthritis (Peterson, 1994a) and followed

by rheumatic (Reiter's syndrome, post-infectious arthritis) (Peterson, 1994b) and neurological complications [Guillain-Barré syndrome, (GBS)] (Nachamkin, 2002; Yuki *et al.,* 2004; 2005; Yuki & Koga, 2006). GBS is an acute, post-infectious immune-mediated paralytic disorder affecting the peripheral nervous system, and is the most common cause of acute flaccid paralysis in the post-polio era (Nachamkin, 2002). Recent studies have revealed that carbohydrate mimicry of *C. jejuni* lipooligosaccharide (LOS) by the human ganglioside is an important cause of the syndrome (Yuki *et al.,* 2004; 2005; Yuki & Koga, 2006).

C. fetus is rarely found as a cause of enteritis but is often isolated in systemic infections. The number of systemic infections observed with *C. fetus* indeed exceeds the number due to thermotolerant *Campylobacter* species such as *C. jejuni* and *C. coli* (Moore *et al.,* 2005). However, more than half of the patients harbour an underlying disease (diabetes, cirrhosis, cancer, immunosupression, HIV infection). In contrast to cattle and sheep, *C. fetus* rarely induces abortions in humans (Sauerwein *et al.,* 1993; van Bergen *et al.,* 2005). The likelihood of bacteremia during infection with *C. fetus* is increased nearly 1,000-fold over that due to *C. jejuni* (Blaser, 1986). Bacteremia may result in lesions in organs distant from the gastrointestinal tract. A number of tissues can be involved, especially the vascular endothelium, bones, joints and meninges (Moore *et al.,* 2005). *Campylobacter fetus* infections must be treated vigorously with antibiotics because of a bad prognosis. In a survey of more than 100 cases, death occurred in 15% of the cases, one-third being attributable to the infection, and a relapse occurred in 10% (Moore *et al.,* 2005).

3.3. Pathogenicity and virulence factors of *Campylobacter jejuni*

Since the association of *Campylobacter* with human enteric disease, a reasonable understanding of the general clinical, microbiological, and epidemiological aspects of infection has been achieved. However, the molecular mechanisms involved in pathogenesis are still rather poorly understood. Few of the factors and/or virulence determinants required to establish an infection and to cause disease have a proven role. This chapter reviews the information available for major enteropathogenic species, *C. jejuni*. The pathogenicity and virulence factors of *C. jejuni* are presented in Table 1.

Campylobacter jejuni pathogenicity determinants are generally not well characterized, and some are rather controversially discussed. Potential reasons underlying this relative paucity of understanding of *C. jejuni* pathogenesis include initial difficulties at molecular genetic manipulations, interstrain variability in virulence, and the lack of an effective animal model of human enteric infection. As research in this area is intensifying with the availability of genome sequences of seven *Campylobacter* strains, a clear picture of *Campylobacter* pathogenesis is awaited.

Table 1. Pathogenicity and virulence factors of *Campylobacter jejuni.*

Name	Gene	Function	References
Pathogenicity factors			
Chemotaxin	*cheY*	detection of chemical gradients, colonization	Yao *et al.,* 1997; Hendrixson & DiRita, 2004
Iron acquisition and its regulation factors	*ceuB,C,D, E, crfA, feoB, fur*	iron acquisition and its regulation, colonization, intracellular survival	van Vliet *et al.,* 2002; Palyada *et al.,* 2004; Naikare *et al.,* 2006
Stringent response regulator SpoT	*spoT*	growth and survival under low CO_2/high O_2, rifampicin resistance, adherence, invasion, intraepithelial survival	Gaynor *et al.,* 2005
Superoxide dimutase	*sodB*	defence against oxidative damage, intraepithelial survival, intramacrophage persistence	Pesci *et al.,* 1994; Purdy & Park, 1994; Grant & Park, 1995; Day *et al.,* 2000
Catalase	*katA*		
Flagellum	*flaA, flaB* (more than 50 genes)	motility, colonization, invasion	Guerry *et al.,* 1990; Yao *et al.,* 1994; Parkhill *et al*., 2000
Flagellum export apparatus	*flhA,B, fliF,G,H,I,M, NO,P,Q,R*	flagellar assembly, Cia secretion, FlaC secretion	Hendrixson & DiRita, 2003; Konkel *et al.,* 2004; Song *et al.,* 2004; Wosten *et al.,* 2004
Adhesins	*cadF, jlpA, peb1*	adherence	Konkel *et al.,* 1997; 1999a; 2005; Pei *et al.,* 1998; Jin *et al.,* 2001; 2003
Lipooligosaccharide (LOS) and its sialic acid moieties	LOS biosynthe-sis locus, variable	immunogenicity, serum resistance, induction of GBS by human ganglioside mimicry	Guerry *et al.,* 2000; 2002; Gilbert *et al.,* 2002; Yuki *et al.,* 2004; 2005
Capsular polysaccharide (CPS)	*cps* locus, variable	evasion of host immune responses, serum resistance, adherence, invasion	Karlyshev *et al.,* 2000; Bacon *et al.,* 2001; Karlyshev *et al.,* 2005b
O-linked flagellar glycosylation system	flagellar glycosyl. locus, variable ~50 genes	flagellar glycosylation and assembly, auto-agglutination, evasion of host immune responses	Thibault *et al.,* 2001; Golden & Acheson, 2002; Logan *et al.,* 2002; Guerry *et al*., 2006
N-linked general glycosylation system	*pgl* locus	glycosylation of >30 proteins, colonization, adherence, invasion	Szymanski *et al.,* 1999; 2002; Linton *et al.,* 2002; 2005; Jones *et al.,* 2004
Virulence factors			
Campylobacter invasion antigens (Cia proteins)	*ciaB,* not determined	invasion, unknown	Konkel *et al.,* 1999b; 2004
FlaC	*flaC*	invasion	Song *et al.,* 2004
pVir	*comB3, virB11*	homolog of type IV secretion system, adhesion, invasion	Bacon *et al.,* 2000; Bacon *et al.,* 2002
Cytolethal Distending Toxin	*cdtA,B,C*	host cell cycle arrest in G_2/M phase, IL-8 induction	Pickett *et al.,* 1996; Hickey *et al.,* 1999; Lara-Tajero & Galan, 2000; 2002

Campylobacter jejuni and *Helicobacter pylori* are closely related at the 16S rRNA phylogeny level, share many biological properties, and due to strong similarities, the latter was previously classified within the *Campylobacter* genus. However, the genome sequence analysis revealed that despite a close phylogenetic relationship, similarities between these

two species are mainly confined to the housekeeping functions. Only 55.4% of *C. jejuni* genes have orthologs in *H. pylori* (Parkhill *et al*., 2000). In most functions related to survival, transmission and pathogenesis, the organisms have remarkably little in common. Genes encoding for well-established *H. pylori* virulence factors, namely vacuolating cytotoxin VacA, the urease, nickel uptake systems, the adhesins Bab and Alp, the *cag* pathogenicity island and the large *hop* gene family encoding outer membrane proteins of *H. pylori* (reviewed in Kusters *et al.,* 2006), are absent in *C. jejuni* (Parkhill *et al*., 2000). However, pathogenicity genes present in both species include functions for chemotaxis, iron acquisition, and motility. The high degree of similarity in housekeeping genes on the one hand, and the striking diversities in virulence factor equipment on the other, provide evidence that although both species evolved from a relatively close common ancestor, selective pressure has driven profound evolutionary changes to create two very different and specific pathogens appropriate to their niches (Parkhill *et al*., 2000).

Campylobacter jejuni is a classical food-borne pathogen, in association with contaminated food or water it enters the host intestine *via* the stomach acid barrier and colonizes the distal ileum and colon of humans. Following colonization of the mucus and adhesion to the intestinal cell surface, *C. jejuni* perturbs the normal absorptive capacity of the intestine by damaging epithelial cell function either directly by cell invasion and/or the production of toxins, or indirectly *via* initiation of an inflammatory response (Ketley, 1997; Wooldrige & Ketley, 1997). As effective colonization requires chemotaxis, *C. jejuni* has evolved mechanisms to detect chemical gradients, which provide appropriate directionality towards mucus. Chemically mutagenized, nonchemotactic mutants of *C. jejuni* fail to colonize the suckling mice intestine (Takata *et al.,* 1992). Furthermore, *C. jejuni cheY* null mutants, displaying a nonchemotactic phenotype, are unable to colonize chicks (Hendrixson & DiRita, 2004) and mice or cause symptoms in infected ferrets (Yao *et al.,* 1997). Thus, the chemotactic response of *C. jejuni* appears to be important in directing the organism to specific sites in the host's intestinal tract.

Acquisition of iron is another important pathogenicity factor for bacteria to cope with the severe iron limitation that occurs in their host environment (Braun & Killmann, 1999). No siderophores (small iron scavenger molecules) have so far been identified in *C. jejuni* (Field *et al.,* 1986; Pickett *et al.,* 1992), which is in agreement with the absence of putative genes encoding these factors in the *C. jejuni* genome sequences (Parkhill *et al*., 2000; Fouts *et al*., 2005; Hofreuter *et al.,* 2006). However, it has been reported that *C. jejuni* makes use of exogenous siderophores produced by other bacteria or the host (*e.g.* ferrichrome, enterobactin, and heme compounds) (Field *et al.,* 1986; Palyada *et al.,* 2004; Ridley *et al.,* 2006). Several putative uptake systems for these siderophores have been identified in the genomes of *C. jejuni*, and some of them have been functionally characterized (reviewed in

van Vliet *et al.,* 2002). Recently presented data indicate the requirement of both ferric-siderophore and ferrous iron transport systems for the colonization of the chick and highlight the importance of ferrous iron acquisition in the pathogenesis of *C. jejuni* (Palyada *et al.,* 2004; Naikare *et al.,* 2006).

Campylobacter jejuni is natural zoonotic, and therefore factors involved in cell survival and resistance to physiological stress are important for its successful transmission and infection. *Campylobacter jejuni* responds very actively to temperature shifts from 37°C to 42°C, which may occur during transmission between different hosts, humans and birds, respectively. The global regulatory responses observed indicate that bacteria are able to adapt rapidly to new environments (Konkel *et al.,* 1998; Stintzi *et al.,* 2003). Recently, a critical role for the *C. jejuni* stringent response, regulated by *spoT*, in stationary phase survival, growth and survival under low CO_2/high O_2 conditions, and rifampicin resistance has been shown. Detailed analyses of a *C. jejuni* Δ*spoT* mutant revealed that the stringent response is in addition required for adherence, invasion, and intracellular survival in human epithelial cells (Gaynor *et al.,* 2005). The superoxide dismutase protein SodB, the main component of the *C. jejuni* defense against oxidative damage, is also known to have a potential role in intraepithelial survival (Pesci *et al.,* 1994; Purdy & Park 1994; Park, 1999). The identification of the gene encoding catalase, *katA* (Grant & Park; 1995), indicates that *C. jejuni* may have other determinants that form part of a defense system against oxidative stress. In agreement to this, a role of catalase in *C. jejuni* intra-macrophage persistence has been reported (Day *et al.,* 2000).

A combination of the unique characteristics of the flagellum and the spiral shape of the bacterial cell has been reported to give *C. jejuni* an unusually high level of motility (Ferrero & Lee, 1988). This motility helps the bacteria to overcome the clearing movement of peristalsis in the gut and enables them to enter and cross the viscous mucous layer covering the epithelium of the small and large intestine (Lee *et al.,* 1986). The flagellum is one of the most intensively investigated pathogenicity determinant of *C. jejuni*. It is composed of basal body, hook and filament. The filament consists of multimers of flagellin and is attached by the hook protein to the basal body, which is embedded in the membrane and serves as a motor for rotation (Wassenaar & Blaser, 1999). The flagellar filament is comprised of two proteins, FlaA and FlaB (Guerry *et al.,* 1990; Nuijten *et al.,* 1990). Both are synthesized concomitantly, but *flaA,* which is regulated by σ^{28} (Guerry *et al.,* 1990), is expressed at much higher levels than *flaB*, which is regulated by σ^{54} (Alm *et al.,* 1993; Wassenaar *et al.,* 1994; Hendrixson *et al.,* 2001). The FlgS-FlgR two-component regulatory system is essential for flagellar biosynthesis (Hendrixson & DiRita, 2003; Wosten *et al.,* 2004). Moreover, transcription of σ^{54}-dependent but not σ^{28}-dependent flagellar genes in *C. jejuni* is associated with formation of the flagellar secretory apparatus, encoded in part by *flhA*,*B*, *fliP*, *fliR* genes (Hendrixson &

DiRita, 2003). The unsheathed flagellum exhibits phase (Caldwell *et al.,* 1985; Diker *et al.,* 1992; Park *et al.,* 2000) and antigenic variation (Harris *et al.,* 1987; Alm *et al.,* 1992). Furthermore, human volunteers dosed with a mixture of motile and nonmotile variants of *C. jejuni* excreted with stool only motile bacteria (Black *et al.,* 1988). The flagellum has been shown to be required for colonization in a number of animal models (Morooka *et al.,* 1985; Pavlovskis *et al.,* 1991; Nachamkin *et al.,* 1993; Wassenaar *et al.,* 1993; Hendrixson & DiRita, 2004). It has also been reported to play an active role in the invasion of epithelial cells (Wassenaar *et al.,* 1991; Grant *et al.,* 1993; Yao *et al.,* 1994) and, surprisingly, may have a role in adhesion (Yao *et al.,* 1994). Thus the motility, needed to reach the intestinal cells and to establish close contact, is not the only consequence of flagellation.

Campylobacter jejuni can either survive as free-living bacteria in the mucous layer or invade the gastrointestinal epithelium. Prior to invasion, the bacteria attach to the epithelial cells (Wassenaar & Blaser, 1999). Adherence of *C. jejuni* is a multifactorial event in which several binding factors may be required to bind to their respective receptors to achieve an efficient interaction with the host. The precise mechanism underlying *C. jejuni* adhesion to host cells has not been defined but a number of putative bacterial adhesins have been recognized. To date, the best-characterized *C. jejuni* adhesins are the *Campylobacter* adhesin to fibronectin (CadF) (Konkel *et al.,* 1997; 2005), the *jejuni* lipoprotein A (JlpA) (Jin *et al.,* 2001; 2003) and the periplasmic binding protein PEB1 (Pei at *al.,* 1998). The CadF protein has been shown to mediate the binding of *C. jejuni* to fibronectin and to promote bacteria-host cell interactions (Konkel *et al.,* 1997; 1999a; Monteville *et al.,* 2003). A mutation in *jlpA* gene results in 19% reduction in adherence when compared to the *C. jejuni* wild-type isolate, but has no effect on *C. jejuni* invasion. In addition, pre-treatment of Hep-2 cells with recombinant JlpA reduces binding of *C. jejuni* to the cells in a dose-dependent fashion (Jin *et al.,* 2001). Ablation of PEB1 affects adherence to and invasion of epithelial cells as well as colonization of mice (Pei *at al.,* 1998). Other molecules proposed to function as adhesins include the flagellum, lipooligosacharide (LOS) (McSweegan & Walker, 1986; Fry *et al.,* 2000), the major outer membrane protein (MOMP, also called OmpE) (Moser *et al.,* 1997; Schroder & Moser, 1997) and P95 (Kelle *et al.,* 1998), but their adhesive properties are still not well characterized.

The capability to translocate across the cell barrier is considered an important virulence attribute for some microbial pathogens as it allows access to underlying tissues and could promote their dissemination throughout the host (Konkel *et al.,* 2001). The advantages for *C. jejuni* reaching the underlying tissue and submucosa include access to a different set of cellular molecules that serve as receptors and the fact that the bacteria are no longer subject to peristaltic action of the intestine. There exists evidence both for paracellular passage (Monteville & Konkel, 2002; Chen *et al.,* 2006) and M-cell transcytosis of *C. jejuni*

(Walker *et al.,* 1988; 1992; Kopecko *et al.,* 2001). It has been shown that basolateral infection with *C. jejuni* causes a more rapid decrease in transepithelial electrical resistance (TER), comparable redistribution of tight-junction proteins, and secretion of more interleukin 8 (IL-8) than infection from the apical surface (Chen *et al.,* 2006). In addition, *C. jejuni* preferentially enters polarized cells *via* the basolateral membrane (Monteville & Konkel, 2002). Moreover, MacCallum and co-workers (MacCallum *et al.,* 2005) proposed that inhibition of absorptive cell function, changes in epithelial resistance and rearrangement of tight junctional proteins such as occludin represent a potential diarrheal mechanism of *C. jejuni*.

Active participation of bacterial pathogens in the translocation of specific virulence factors into host cells for subverting cellular processes is a paradigm among a diverse group of bacterial pathogens, including *C. jejuni* (Konkel *et al.,* 2004). *Campylobacter jejuni* synthesizes a set of proteins during co-culture with epithelial cells, some of which are secreted (Konkel & Cieplak, 1992; Konkel *et al.,* 1993; Konkel *et al.,* 1999b). The secreted proteins have been collectively referred to as *Campylobacter* invasion antigens (Cia proteins) (Konkel *et al.,* 1999b). The functions of the secreted proteins are not yet known; however, insertional mutagenesis of *ciaB* encoding the secreted protein CiaB results in deficiency in the secretion of all Cia proteins and in a significant reduction in the number of internalized bacteria (Konkel *et al.,* 1999b). *Campylobacter jejuni* does not encode a classical type III secretion system (T3SS) (Parkhill *et al*., 2000; Fouts *et al*., 2005; Hofreuter *et al.,* 2006), thus, the flagellar export system has been proposed to secrete both flagellar and non-flagellar proteins (Konkel *et al.,* 2004; Song *et al.,* 2004). Konkel and co-workers (Konkel *et al.,* 2004) reported that the intact flagellar structure, containing the basal body, the hook and at least a partial filament, is required for CiaB protein secretion. They also showed that the flagellar export apparatus serves as the export apparatus for Cia proteins. Another lately characterized *C. jejuni* protein, FlaC, also requires the functional flagellar apparatus for its secretion. *FlaC* mutants form a morphologically normal flagellum and are highly motile, but are defective in invasion of epithelial cells (Song *et al.,* 2004).

In addition, the homologs of other secretion systems, namely the type IV secretion system (T4SS), have been identified on a large plasmid (pVir) in *C. jejuni* 81-176 (Bacon *et al.,* 2000). In general, T4SSs are widespread in gram-negative bacteria and are involved in interbacterial DNA-transfer and protein transport, contributing to virulence (reviewed in Backert & Meyer, 2006). pVir is a 37.5-kb plasmid containing 54 predicted open reading frames (Bacon *et al.,* 2002). Mutation of the plasmid genes *comB3* and *virB11*, which encode two putative homologs of T4SS components, reduces adhesion and invasion *in vitro* comparing with the parental strain. Furthermore, the virulence of the *virB11* mutant is reduced in the ferret diarrheal model. However, transfer of the plasmid to the sequenced

strain, NCTC 11168, does not increase the relatively low invasiveness of this isolate (Bacon *et al.,* 2002).

In the last few years, a variety of toxic activities have been attributed to *C. jejuni* (reviewed in Wassenaar, 1997; Pickett, 2000). However, the cytolethal distending toxin CDT is the only verified *C. jejuni* toxin reported so far (Crushell *et al.,* 2004). Apart from CDT, hemolysin and putative phospholipase A, no other toxin-like homologs could be identified in the sequenced strains (Parkhill *et al*., 2000; Fouts *et al*., 2005; Hofreuter *et al*., 2006) still, reports of a novel toxin-mediated activity continue to appear (Lee *et al.,* 2000). CDT is encoded by three adjacent genes termed *cdtA*, *cdtB,* and *cdtC* (Pickett *et al.,* 1996). All the *C. jejuni* isolates tested possess *cdt* genes, but the levels of expressed toxin activities are strain-dependent (Eyigor *et al.,* 1999; Bang *et al.,* 2001; AbuOun *et al.,* 2005). CDT is a classical AB toxin composed of CdtB as the enzymatically active (A) subunit and of CdtA and CdtC as the heterodimeric (B) subunit, which is required for the delivery of CdtB into the target cells (Lara-Tajero & Galan, 2001). The CdtB exerts its effect as DNAase (Elwell & Dreyfus, 2000; Lara-Tajero & Galan, 2000). The chromatin disruption observed after transient expression of CdtB in cultured cells indicates that the cell death is caused by Cdt-mediated DNA degradation, which is in turn responsible for the cell cycle arrest in G_2/M phase (Lara-Tajero & Galan, 2000). The effects of CDT on cultured cells are profound, but little is known regarding the functional role of the toxin for bacterial pathogenesis *in vivo*. It is likely that CDT exerts its effect on the cells that normally undergo continuous replication, such as those that line the intestinal epithelium or those associated with the immune system. By inducing cell cycle arrest, the *C. jejuni* CDT toxin could influence the renewal and developmental process of epithelial cells to facilitate intestinal colonization, perhaps by increasing the number of cells that could be permissive for bacterial attachment. Likewise, CDT could influence the activity of cells of the immune system such as B or T cells by interfering with their developmental maturation into effector cells (Lara-Tajero & Galan, 2002). In agreement to this assumptions, Fox and co-workers (Fox *et al.,* 2004) demonstrated that the *C. jejuni cdtB* mutant is less efficient than the wild-type in colonizing mice, but not nuclear factor κB (NF-κB)-deficient mice. Despite 100% colonization of NF-κB-deficient mice, the *C. jejuni cdtB* mutant induce significantly less gastritis. In contrast, the recent isolation of CDT-negative strains from cases of human enteric disease raised questions about the role of active CDT in the pathogenesis of campylobacteriosis (AbuOun *et al.,* 2005).

3.4. Host cell factors involved in *Campylobacter jejuni* invasion

Microbial internalization in host cells can occur by several described mechanisms. The co-evolution of bacterial pathogens and their host has contributed to the development of very complex and sophisticated functional pathogen-host interfaces. Thus, well-adapted pathogens have evolved a variety of strategies to manipulate host cell functions precisely and initiate disease. Many invasive bacterial pathogens are known to interact with host cells *via* intimate biochemical crosstalk, stimulating signaling cascades in both the bacterium and the host that ultimately trigger rearrangements of the host cytoskeleton and cause internalization of the pathogen (reviewed in Knodler *et al.,* 2001; Pizarro-Cerda & Cossart, 2006). Bacterial invasion of human epithelial cells *in vivo* ultimately results in cellular injury, the consequent loss of cellular function and diarrhea. Therefore, the invasion of gut tissue cells has been proposed as an important pathogenic mechanism for *C. jejuni*. Early studies of intestinal biopsies from patients (van Spreeuwel *et al*., 1985), experimental studies in primates (Russel *et al.,* 1993) and other experimental model animals (Babakhani *et al.,* 1993) together with *in vitro* infection experiments with cultured human intestinal epithelial cells (De Melo *et al.,* 1989; Grant *et al.,* 1993; Oelschlaeger *et al.,* 1993) supported this hypothesis. Histological examination has indicated pathology primarily in the colon (Black *et al*., 1988; Babakhani & Jones, 1993; Russell *et al.,* 1993), with *C. jejuni* being observed within intestinal crypts, both close to the cell surface and inside intestinal epithelial cells (Babakhani *et al.,* 1993; Babakhani & Jones, 1993; Russell *et al.,* 1993) and mononuclear phagocytes infiltrating the submucosal lining (van Spreeuwel *et al*., 1985). Since then, the invasion of host target cells has been indicated as one of the primary reasons of tissue damage caused by *C. jejuni in vivo* (reviewed in Kopecko *et al.,* 2001). As an *in vitro* model system to study *C. jejuni* invasion intestinal epithelial cells (INT-407) and *C. jejuni* 81-176 strain are used world-wide. 81-176 is a clinical isolate exhibiting a high level of invasion of host cells and its entire genome sequence has been recently determined (Hu & Kopecko, 1999; Hu *et al.,* 2006a; Hofreuter *et al.,* 2006).

Common signal transduction pathways in eukaryotic cells are initiated *via* activation of membrane-associated receptor protein kinases, which results in specific protein phosphorylation events, thereby activating host proteins (Kopecko *et al.,* 2001). Recent studies have shown that inhibition of protein tyrosine kinases markedly reduces *C. jejuni* invasion (Wooldridge *et al.,* 1996; Biswas *et al*., 2000; 2004; Hu *et al.,* 2006a) and that *C. jejuni* infection induces tyrosine phosphorylation of several host cell proteins (Biswas *et al*., 2004; Hu *et al.,* 2006a). Additionally, the heterotrimeric G proteins of the $G\alpha_i$ subfamily have also been implicated in various host signaling events necessary for *C. jejuni* entry (Wooldridge *et al.,* 1996). Furthermore, host cells normally respond to transient increases in

intracellular free Ca^{2+} levels by rearranging the cytoskeleton or by upregulating specific nuclear gene transcription machineries. *Campylobacter jejuni* induces Ca^{2+} release from host intracellular stores, which is essential for its uptake into the host cells (Hu *et al.,* 2005). Numerous reports suggest microtubule-dependent (actin-filament-independent) and/or actin-filament-dependent mechanisms by which *C. jejuni* invades gut tissue cells but no consensus has been established (Oelschlaeger *et al.,* 1993; Hu & Kopecko, 1999; Biswas *et al.,* 2000, 2003; Monteville *et al.,* 2003). Hu and Kopecko (Hu & Kopecko, 1999) reported that a successful interaction between *C. jejuni* ligand and host cell receptor activates dynein bound in caveolae leading to invagination of the dynein-bound membrane, resulting in engulfment of the adjacent adherent bacterium (Hu & Kopecko, 1999). Once internalized, *C. jejuni* can survive for extended periods of time within epithelial cells and ultimately induce a cytotoxic response *in vitro* (Konkel *et al.,* 1992; Day *et al.,* 2000). Intracellular survival may enhance its ability to evade the host immune system, cause relapse of the acute infection, and establish long-term persistent infections (Lastovica, 1996; Day *et al.,* 2000).

Although major pathogenicity and virulence determinants of *C. jejuni,* shown in Table 1*,* represent bacterial factors which may also be involved in host cell invasion, their exact role is not well characterized and the mechanism by which *C. jejuni* triggers eukaryotic cell entry is still poorly understood. Very limited information is currently available concerning not only bacterial but also the host cell factors involved in this process (Biswas *et al.,* 2004).

3.5. Host inflammatory responses

A feature of *C. jejuni* induced pathology is its ability to induce inflammatory diarrhea. Although very little is known about the mechanisms by which *C. jejuni* induces diarrhea, it is likely that its capability to stimulate the production of proinflammatory cytokines plays a central role in this process. Upon *C. jejuni* infection, increased expression and release of proinflammatory cytokines that are dependent on NF-κB and mitogen-activated protein (MAP) kinase signaling pathways has been detected in cultured epithelial cells (Hickey *et al.,* 1999; 2000; Mellits *et al.,* 2002; Hu & Hickey, 2005; Watson & Galan, 2005; Chen *et al.,* 2006; Johanesen & Dwinell, 2006) monocytes (Jones *et al.,* 2003; Siegesmund *et al.,* 2004; Hickey *et al.,* 2005) and dendritic cells (DCs) (Hu *et al.,* 2006b).

The intestinal epithelium forms not only a crucial physical barrier between the body and the luminal environment but also, by producing a defined set of chemoattractant molecules, actively participate in innate and adaptive immune surveillance, thus forming the primary defense against many mucosal enteropathogens. During *C. jejuni* infection *in vitro*, human epithelial cells liberate interleukin 8 (IL-8), a potent proinflammatory cytokine (Hickey *et al.,* 1999; 2000; Johanesen & Dwinell, 2006). Such innate immune response may help limit

the extent of the infection but may be partially responsible for the symptoms. Additionally, *C. jejuni* infection induces transcription and secretion of growth-related oncogene α (GROα) and γ (GROγ), macrophage inflammatory protein 1 α (MIP-1α) and 3 α, (MIP-3α), monocyte chemoattractant protein 1 (MCP-1) and gamma interferon-inducible protein 10 (γIP-10) in epithelial cells *in vitro* (Hu & Hickey, 2005; Johanesen & Dwinell, 2006). Signaling pathways activated in *C. jejuni*-infected epithelial cells share features with host innate immune responses mediated by members of the Toll-like receptor (TLR) and nucleotide-binding oligomerization domain (NOD) family of proteins that recognize conserved microbial components (Akira & Takeda, 2004; Chen *et al.,* 2006; Johanesen & Dwinell, 2006). Chemokine secretion from epithelial cells requires activation of the transcription factor NF-κB, live bacteria and is highly correlated with the efficiency of *C. jejuni* invasion *in vitro* (Johanesen & Dwinell, 2006). However, *C. jejuni* lipooligosaccharide and flagellin are not potent TLR ligands (Hu & Hickey, 2005; Watson & Galan, 2005; Johanesen & Dwinell, 2006) in opposition to lipopolysaccharide and flagellin from *Escherichia coli* (Andersen-Nissen *et al.,* 2005) and *Salmonella enterica* serovar Typhimurium (Gewirtz *et al.,* 2001), respectively. Johanesen & Dwinell (Johanesen & Dwinell, 2006) proposed a novel mechanism of proinflammatory chemokine production whereby *C. jejuni* avoids signaling trough TLR and speculated that intracellular pattern recognition receptors, such as NOD proteins (Viala *et al.,* 2004), may be involved in *C. jejuni* signaling in the host. In contrast, Watson and co-workers (Watson *et al.,* 2007) have recently shown that mice deficient in the adaptor protein myeloid differentiation factor (MyD88), which is required for signaling trough most TLRs (Akira *et al.,* 2001), could be efficiently and persistently colonized by *C. jejuni*, indicating the important role of TLRs in the control of *C. jejuni* infections. One *C. jejuni*-derived factor that can induce IL-8 production from epithelial cells *in vitro* is CDT (Hickey *et al.,* 1999), but CDT is not required for induction of IL-8 release from cultured INT-407 cells by live *C. jejuni* infection, indicating that IL-8 may be induced by other stimuli (Hickey *et al.,* 2000). Thus, the stimulatory signal by which *C. jejuni* triggers innate immune responses in epithelial cells remains to be investigated. The up-regulation of chemoattractants, whose major known function is to attract cells important for antigen presentation and the development of the host adaptive immune response, implicates the epithelium as a key regulator of mucosal immunity in *C. jejuni* infection. Most bacteria eventually will be phagocytosed by leukocytes or macrophages in the reticuloendothelial system *in viv*o (Wassenaar & Blaser 1999). Survival of *C. jejuni* within monocytes has been observed *in vitro* for up to seven days and this intracellular survival has been used to explain the complication of long-term bacteremia, since such a niche could provide the bacteria with an immunologically privileged site (Kiehlbauch *et al.,* 1985; Hickey *et al.,* 2005). However, in other *in vitro* studies the macrophages (Wassenaar *et al.,* 1997) and DCs (Hu *et al.,* 2006b) were able to kill all tested strains with high efficiency, findings in

agreement with the self-limiting nature of most *C. jejuni* infections. Thus, DCs may play a key role in antigen processing and directing the development of the adaptive immune response in campylobacteriosis (Hu *et al.,* 2006b). As demonstrated by the higher incidence, severity, and relapse of *C. jejuni* infections in HIV-infected patients, adaptive immunity is important to limit and clear the bacterial infection (Sorvillo *et al.,* 1991; Morpeth & Thielman, 2006). Since epidemiological observations in developing countries suggest that the high-level exposure to *C. jejuni* leads to immunity with protection from disease, the development of a vaccine against *C. jejuni* seems feasible (Scott, 1997). However, it has been hindered by the lack of understanding of the virulence mechanisms, antigenic complexity of these organisms and by the theoretical risk of triggering immunological sequel, such as GBS. In view of these findings, a vaccine should be carefully designed and a subunit vaccine may be preferable to a whole cell vaccine (Kopecko, 1997; Girard *et al.,* 2006).

3.6. *Campylobacter jejuni* surface structures and their role in evasion of host immune responses

Generation of antigenic variation is one of the mechanisms enabling bacteria to express new variants of surface components and evade host immune responses during infection (Finlay & Falkow, 1997; Abramovitch *et al.,* 2006; Pizarro-Cerda & Cossart, 2006). Antigenic variations can be achieved by several mechanisms including slipped-strand mispairing, exchange of genes and entire clusters by horizontal transfer or contingency gene variations. Rearrangement of genetic loci involved in expression of major antigens, an efficient mechanism for generation of diversity, is employed by a wide range of microbes (Henderson *et al.,* 1999; Tu *et al.,* 2005).

Campylobacter jejuni possesses enormous capacity to produce a variety of carbohydrates. Presence of several cell surface glycoconjugates such as a capsule and *N*-linked glycosylation pathway, as well as the lipooligosacharide (LOS) and the *O*-linked glycosylation system that decorates the flagellum has recently been reported (reviewed in Szymanski *et al.,* 2003; Karlyshev *et al.,* 2005a; Szymanski & Wren, 2005). Interestingly, *C. jejuni* genome has several hypervariable homopolymeric repeats responsible for slipped-strand mispairing and phase variation in glycan moieties present in LOS, capsule and flagellum (Parkhill *et al*., 2000; Fouts *et al.,* 2005; Hofreuter *et al.,* 2006). LOS and capsule are important surface structures in *C. jejuni* that function in the interactions of the organism with the environment. Modulation of the expression of these surface structures is likely to be important *C. jejuni* strategy for avoiding host defenses and possibly adaptation to dynamic and hostile environments (Karlyshev *et al.,* 2005a). Various *C. jejuni* LOS structures have been described (reviewed in Moran *et al.,* 2000). The degree of genetic variation that

generates the diversity apparent in LOS structure signifies its functional importance for *C. jejuni* (Karlyshev *et al.,* 2005a). The LOS sialic acid (NeuNAc) moieties affect immunogenicity and serum resistance of *C. jejuni* (Guerry *et al.,* 2000; 2002) and resemble human gangliosides (reviewed in Yuki *et al.,* 2005; Yuki & Koga, 2006). This molecular mimicry and the subsequent generation of cross reacting antibodies against gangliosides are though to play a role in the ability of some strains of *C. jejuni* to induce GBS (Gilbert *et al.,* 2002; Godschalk *et al.,* 2004; Yuki *et al.,* 2004, 2005; Yuki & Koga, 2006). Nevertheless, given that most enteric infections with *C. jejuni* strains expressing ganglioside mimics do not result in GBS (Nachamkin *et al.,* 1999; Nachamkin, 2002), suggest that other unknown host and/or bacterial factors are also essential. However, the latter factors remain unknown (Karlysev *et al.,* 2005a).

Another class of *C. jejuni* cell surface molecules with a potential for structural variation is capsular polysaccharide (CPS) (Karlyshev *et al.,* 2005a). It is the major antigenic component of the classical Penner serotyping system distinguishing *C. jejuni* into more than 60 groups (Moran & Penner, 1999; Karlyshev *et al.,* 2000). CPS undergoes antigenic variation at high frequency (Bacon *et al.,* 2001). There exist multiple genetic mechanisms underlying the structural heterogeneity in CPS including exchange of capsular genes and entire clusters by horizontal transfer, contingency gene variation, gene duplication, deletion and fusion (Karlyshev *et al.,* 2005b). Variation in the CPS structure may be essential for escape from host immune surveillance and this is supported by the finding that loss of CPS is associated with attenuated *C. jejuni* virulence *in vitro* and in ferret diarrheal disease model (Bacon *et al.,* 2001). Moreover, the CPS of *C. jejuni* has been reported to have a role in increasing surface hydrophilicity and serum resistance (Bacon *et al.,* 2001).

Campylobacter jejuni flagellins are among the most heavily glycosylated prokaryotic proteins described (Thibault *et al.,* 2001; Logan *et al.,* 2002). Recently, the complete flagellin *O*-linked glycosylation locus of *C. jejuni* has been characterized (Guerry *et al*., 2006; Mc Nally *et al.,* 2006). Although the glycans show variability among strains and can confer serospecificity (Logan *et al.,* 2002), the major carbohydrate modifications on both flagellins are pseudaminic acid (Pse5NAc7NAc), a sugar that is structurally similar to sialic acid, and its acetamidino derivative (PseAm) (Thibault *et al.,* 2001). Glycosylation is not only essential for flagellar assembly and consequent motility (Linton *et al.,* 2000; Thibault *et al.,* 2001; Logan *et al.,* 2002; Goon *et al.,* 2003) but the glycans on flagellin play also a role in autoagglutination and pathogenesis of *C. jejuni* (Golden & Acheson, 2002; Guerry *et al.,* 2006). The potential for generating structural diversity in the flagellin owing to *O*-linked glycosylation suggest that it enables the bacteria to generate antigenic diversity in this surface exposed and immunodominant protein. This would suggest a role in immune evasion, probably in avian part of the life cycle (Szymanski *et al.,* 2003).

Significantly, unlike loci involved in *C. jejuni* capsule, LOS and flagellin biosynthesis which are highly variable among different strains (Linton *et al.,* 2000; Bacon *et al.,* 2001; Dorrell *et al.,* 2001; Gilbert *et al*., 2002; Guerry *et al.,* 2002; Karlyshev *et al.,* 2002; 2005a; Logan *et al.,* 2002), the *N*-linked glycosylation locus is highly conserved even among some other *Campylobacter* species (Szymanski *et al.,* 2003; Szymanski & Wren, 2005). *Campylobacter jejuni* is unique for a prokaryotic organism with respect to the presence of a general system of *N*-linked protein glycosylation (Pgl), affecting a substantial number of periplasmic and surface proteins (Szymanski *et al.,* 1999; Linton *et al.,* 2002; Wacker *et al.,* 2002; Young *et al.,* 2002; Larsen *et al*., 2004). The proteins glycosylated *via* the *pgl* locus harbour a heptasaccharide motif that contains diacetamidobacillosamine (DAB) (Wacker *et al.,* 2002; Young *et al.,* 2002; Linton *et al.,* 2005; Vijayakumar *et al.,* 2006). The loss of the carbohydrate components appears to cause drastic reduction in reactivity of these glycoproteins with antisera, indicating that the glycosyl moieties may be immunodominant. Moreover, *C. jejuni pgl* mutants have a reduced ability to adhere to and invade human epithelial cells and to colonize the intestinal tract of mice (Szymanski *et al.,* 2002) and chickens (Hendrixson & DiRita, 2004; Jones *et al.,* 2004; Karlyshev *et al.,* 2004), reinforcing the importance of protein glycosylation for the pathogenesis of *C. jejuni* (Szymanski *et al.,* 2002). However, the precise functional contribution of *N*-linked glycosylation to the pathogenesis of *C. jejuni* remains unclear (Larsen *et al.,* 2004). In contrast to the *O*-linked glycan, the relative conservation of the *N*-linked glycan argues against a role in avoidance of host defense for this modification (Szymanski *et al.,* 2003). Possible functions of *N*-linked glycosylation may include protection against proteolytic cleavage, enhancement of protein stability or signals for cellular sorting as has been suggested for analogues eukaryote *N*-linked glycans (Herrmann *et al.,* 1996; Helenius & Aebi, 2001).

3.7. Pathogenicity and virulence factors of *Campylobacter fetus*

The pathogenesis of C. *fetus* disease is even less well defined than that of *C. jejuni* (Graham, 2002). *Campylobacter fetus* is known to possess flagella (McCoy *et al.,* 1975) and *cdt* genes (Asakura *et al.,* 2007) but their role in *C. fetus* pathogenesis has not been investigated yet. *C. fetus* was shown to adhere and invade intestinal epithelial cells but the pathogenicity factors playing a role in these processes are unknown (Graham, 2002). Unlike *C. jejuni*, *C. fetus* expresses a paracrystalline surface layer (S-layer) on its outermost cell surface (Dubreuil *et al.,* 1988; 1990; Fujimoto *et al.,* 1991). S-layer is composed of acidic high-molecular-weight S-layer proteins (SLPs) encoded by up to nine homologous genes (*sapA1* to *sapA8*) in each strain (Dworkin *et al.,* 1995; Garcia *et al.,* 1995; Tu *et al.,* 2001a). The S-layer is the major pathogenicity factor of *C. fetus* (Pei & Blaser, 1990; Blaser & Pei,

1993; Grogono-Thomas *et al.,* 2000). The SLPs play a critical role in *C. fetus* pathogenesis by protecting the bacterium from phagocytosis and serum killing, *via* impairing C3b binding (Blaser *et al.,* 1987; 1988; Blaser & Pei, 1993). Moreover, SLPs are essential for host colonization (Grogono-Thomas *et al.,* 2000). In addition, the SLPs are able to undergo antigenic variation (Dubreuil *et al.,* 1990; Garcia *et al.,* 1995; Wang *et al.,* 1993) by DNA invertion (Dworkin & Blaser, 1996; 1997a; 1997b; Tu *et al.,* 2001b; 2003). Each *sapA* homologue can reciprocally recombine with the others, with rearrangements permitting the creation of new *sapA* homologues within the *sap* locus. This genomic plasticity, based on recombination of homologues units, can result in substantial antigenic variation, beyond that produced by independent genes, leading to a repertoire of great complexity (Tu *et al.,* 2003). Generation of antigenic diversity allows *C. fetus* to escape the immune defense of the host and permits the pathogen to endure an immunologically hostile environment (Blaser & Pei, 1993).

The multiple immune system evasion mechanisms levied by the S-layer undoubtedly facilitate *C. fetus* survival within a host, contributing to the persistence observed in *C. fetus* infections (Neuzil *et al.,* 1994; Tu *et al.,* 2005).

3.8. A model of *Campylobacter jejuni* pathogenesis

The interplay between target cells and variety of pathogenicity factors modulates multiple host responses, leading to enteritis. Figure 2 presents a hypothetical model of *C. jejuni* pathogenesis which is based on articles discussed above.

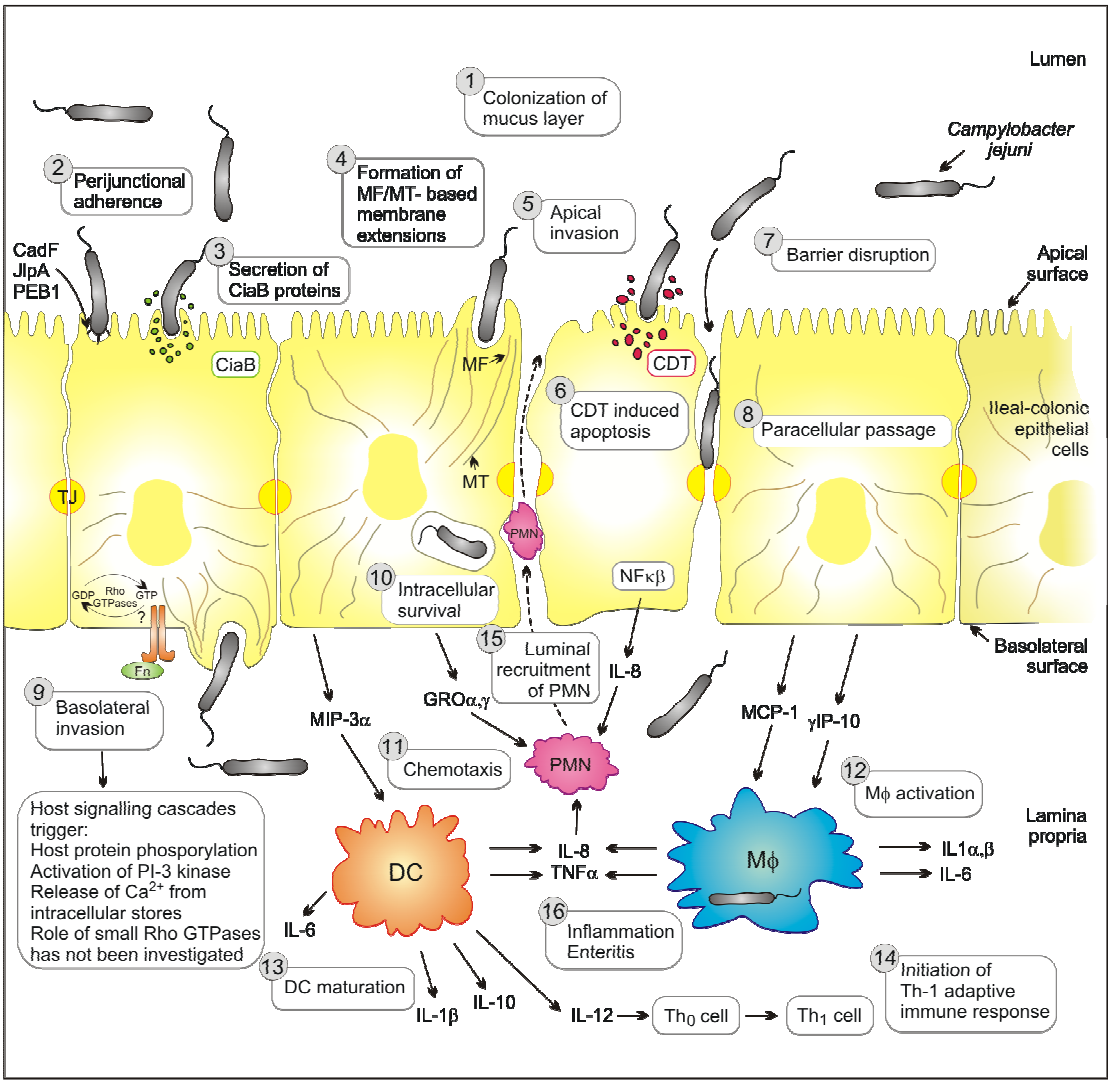


Fig. 2. Hypothetical model of *C. jejuni* pathogenesis. (1) *C. jejuni* initially colonizes the mucous layer of the jejunum and ileum, and then the colon. (2) *C. jejuni* adheres to the apical surface at the perijunctional region of host intestinal epithelial cells, *via* adhesins including CadF, JlpA, and PEB1. (3) Upon contact with cells *C. jejuni* synthesizes and secretes *Campylobacter* invasion antigens (Cia proteins). (4) Host signaling cascades trigger microfilaments (MF) and microtubules (MT) rearrangements. (5) These events are crucial for *C. jejuni* host cells invasion. (6) *C. jejuni* CDT toxin induces host cell cycle arrest, apoptosis and proinflammatory cytokine, IL-8, secretion. (7) Opening of tight junctions (TJ) and disruption of the epithelial barrier by CDT and intracellular *C. jejuni* enable (8) bacterial transcytosis *via* a "leaky" paracellular pathway to (9) the basolateral surface where they continue to invade cells and induce host proteins phosphorylation, activation of PI3 kinase and the release of Ca^{2+} from intracellular stores. (10) *C. jejuni* survive and replicate, at least to some extent, within intracytoplasmic vacuoles. (11) During infection epithelial cells liberate cytokines: IL-8, GROα,γ, MIP-3α, MCP-1, γIP-10 which lead to recruitment of polymorphonuclear leucocytes (PMNs), macrophages (Mϕ), dendritic cells (DCs) and lymphocytes to the site of infection. (12) Activated macrophages release several proinflammatory cytokines, such as IL-1α,β, IL-6, IL-8, TNFα. (13) The IL-12 micro-environment induced by maturated DCs is particularly important for (14) shifting CD4+ helper response into prominent Th1 type which may play a key role in the development of the adaptive immune response in campylobacteriosis. (15) The loss of epithelial integrity may cause the net loss of fluid, which also contains blood, protein, and inflammatory cells, into lumen thus (16) causing diarrhea and enteritis.

3.9. Aim of the study

Campylobacter infections are the leading cause of bacterial diarrhea in the developed world and therefore present a significant challenge to public health. Despite their importance, effective control of *Campylobacter* in the food chain and the design of disease prevention strategies are hindered by a poor understanding of pathogenesis of the organisms. Despite the array of virulence factors identified so far, it has not been possible to develop a vaccine. Further progress in both understanding of biological significance of the *Campylobacter* pathogenicity factors and the nature of the bacterial interactions with the host during invasion process will lead to new strategies for detecting, controlling, and reducing *Campylobacter* infections. This will help to decrease the human illness in the long term and ultimately reduce the economic loss because of lost working hours and clinical testing costs.

The present study was performed to characterize bacterial pathogenicity factors, which are involved in invasion process of *C. jejuni* and *C. fetus.*

1. The first aim of this study was to determine the genetic and functional diversity of CadF protein among *Campylobacter* strains. For this purpose, the expression of CadF proteins of a large number of *C. jejuni* and *C. coli* isolates of human and animal origin should be investigated and the role of CadF in the attachment and internalization of INT-407 epithelial cells should be determined.
2. The next aim was to analyze the interaction of *C. jejuni* with the surface of INT-407 epithelial cells by high resolution field emission scanning electron microscopy (FESEM) and to investigate the functional importance of small Rho GTPase members during host cell entry of *C. jejuni* with use of specific GTPase-modifying toxins, inhibitors, siRNA and GTPase expression constructs. Furthermore, to identify bacterial factors involved in *C. jejuni*-induced GTPase activation several isogenic mutants of *C. jejuni* pathogenicity factors should be tested.
3. Subsequently, host signal transduction events upstream of *C. jejuni*-induced GTPase activation ought to be examined and importance of the $ß_1$ integrins, epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR) and the focal adhesion kinase (FAK) in host cell invasion of *C. jejuni* should be determined, using $ß_1$- and FAK-deficient cell lines, expression constructs and inhibitors.
4. Finally, the role of the surface array protein SapA in infection with *Campylobacter fetus* should be established by SapA cloning, purification and *in vitro* tests as well as, by examination of SapA-non-expressing strains.

The results of this study should provide a detailed understanding of unique mechanism by which *C. jejuni* invade host target cells.

4. Materials & Methods

4.1. *Campylobacter* strains and growth conditions

Campylobacter jejuni, *C. coli* and *C. fetus* wild-type isolates and their isogenic mutants used in this study were provided by cooperation partners and are listed in Table 2.

Table 2. *Campylobacter* strains used in this study.

Strains [a, b]	Origin
***C. jejuni* and *C. coli* wild-type isolates**	
Han 36, 1991, 2371, Han 35, 503, Han 153, K1102/03, av245, av352	Prof. Dr. Thomas Alter, Federal Institute for Risk Assessment, Unit Food Hygiene and Safety Concepts, Berlin, Germany
158/96, 157/96, 73 Di, 100204ZH0021, 151003Z-H0099, 201004ZH0078, 51/89, 230205ZH0017, 230205ZH0018, C130	Dr. Ingrid Hänel, Federal Research Institute for Animal Health, Jena, Germany
Alk1116, G447, G448, G450, G451, G427, Alk1158, G464, G465, G467, Alk1179, Alk1184, Alk1185, Alk1187, G472, G477, G478, G479, G481, G482, Alk1290, Alk1295, G487, G500, G510, G506, Alk1233, Alk1282	Dr. Annette Schliephake, Federal Institute of Saxonia Anhalt (Landesamt für Verbraucher-schutz), Stendal, Germany
CDC 2004-341	Prof. Dr. Omar A. Oyarzabal, Department of Poultry Science, Auburn University, Auburn, USA
RM1221, ATCC43430, ATCC43431, NCTC 11168, RM1849, 81-176, 81-176 pWM1007-*gfp*	Dr. William G. Miller, USDA, ARS, WRRC, Produce Safety and Microbiology Research Unit, Albany, USA
ST3046, 1543/01	Institute of Medical Microbiology, Magdeburg, Germany
***C. jejuni* wild-type isolates and their isogenic mutants**	
F38011 and F38011Δ*cadF*	Prof. Dr. Michael E. Konkel, School of Molecular Biosciences, Center for Biotechnology, Washington State University, Pullman, USA
81116 and 81116Δ*cadF*	Prof. Dr. Jos P. M. van Putten, Department of Infectious Diseases and Immunology, Utrecht University, Utrecht, The Netherlands
84-25, 84-25Δ*kpsS,* 84-25Δ*kpsS/kpsS,* 84-25Δ*waaF,* 84-25Δ*waaF/waaF,* 81-176, 81-176Δ*pEB1A*, 81-176Δ*flaA/B*, 81-176Δ*flhA*	Prof. Martin J. Blaser, Department of Medicine, New York University School of Medicine, New York, USA
81-176Δ*cdtB*	Prof. Patricia Guerry, Enteric Diseases Department, Naval Medical Research Center, Silver Spring, Maryland, USA
***C. fetus* wild-type isolates and their mutants**	
S1-, S1+, S2-, S2+, S3-, S3+	Prof. Shuji Fujimoto, Department of Bacteriology, Faculty of Medicine, Kyushu University, Fukuoka, Japan
MGH 97-2126, MGH 97-3574	Prof. David B. Schauer, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA
5361	Institute of Medical Microbiology, Magdeburg, Germany

[a] Further characteristic of these strains is presented in Table 8 and Table 9 in Results chapter.

[b] Refers to the Table 2 on page 23. Species identification, performed by the cooperation partners, was based on biochemical tests (catalase, oxidase, urease activity, hippurate and indoxyl acetate hydrolysis, and sensitivity to cephalothin and nalidixic acid), and a multiplex PCR assay (Cloak & Fratamico, 2002; Oyarzabal *et al.,* 2005).

All *Campylobacter* strains were grown on *Campylobacter* blood-free selective Agar Base (Oxoid Basingstoke, UK) containing *Campylobacter* growth selective supplement (Oxoid) or, when appropriate, on Mueller-Hinton (MH) agar amended with antibiotics at 37°C under microaerophilic conditions (generated by CampyGen (Oxoid) in AnaeroJar (Oxoid)) for 48 hrs.

***Campylobacter* Blood Free Selective Agar Base**	
per liter	
Nutrient Broth No. 2	25.0 g
Bacteriological charcoal	4.0 g
Casein hydrolysate	3.0 g
Sodium desoxycholate	1.0 g
Ferrous sulphate	0.25 g
Sodium pyruvate	0.25 g
Agar	12.0 g

Mueller-Hinton-Agar	
per liter	
Beef infusion solids	4.0 g
Casein hydrolysate	17.5 g
Starch	1.5 g
Agar	15.0 g

***Campylobacter* Selective Supplement**	
per liter	
Cefoperazone	32.0 mg
Amphotericin B	10.0 mg

Antibiotics	**Final concentration**
Chloramphenicol	4 µg/ml
Tatracycline	10 µg/ml
Kanamycin	20 µg/ml, resp. 200 µg/ml*

* Used for selection of *Campylobacter* cells expressing pWM1007-*gfp* (Miller *et al.,* 2000).

4.2. *Escherichia coli* (*E. coli*) strains and growth conditions

E. coli strains used in this work are presented in Table 3.

Table 3. *E. coli* strains used in this study.

Strain	Genotype
TOP10	F⁻ *mcr*A Δ*(mrr-hsd*RMS-*mcr*BC*)* Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rsp*L (Str^R) *end*A1 *nup*G
BL21	F⁻ *ompT hsdS*$_B$(r^{B-} m^{B-}) *gal dcm*

Cultivation of *E. coli* was carried out on Luria-Bertani (LB) and Yeast tryptone medium 2xYT at 37ºC with shaking at 200 rpm or on LB agar plates. All strains were kept as Brain Heart Infusion (BHI)-20% (v/v) glycerol stock cultures at -70°C.

LB medium [a]	
per liter	
tryptone	10.0 g
yeast extract	5.0 g
NaCl	10.0 g

2xYT	
per liter	
tryptone	16.0 g
yeast extract	10.0 g
NaCl	10.0 g

[a] 18 g/L agar was added to media for preparation of agar plates.

BHI medium	
per liter	
BHI	37.0 g

For selection of antibiotic resistant *E. coli,* sterile-filtered antibiotics were added to the media.

Antibiotics	Final concentration
Ampicillin	100 µg/ml
Kanamycin	30 µg/ml

4.3. Plasmids

Plasmids used in this work are presented in Table 4.

Table 4. Plasmids used in this study.

Plasmid	Marker/characteristics	Origin/References
pRK5	ColE ori, CMV Promoter, MCS, SV40 PolyA, SV40 ori, f1 ori, Amp^R, c-Myc	BD Biosciences, San Diego, USA
pRK5-Rac1-Q61L, pRK5-RhoA-G14V, pRK5-Cdc42-Q61L	pRK5 constructs containing cDNAs of c-Myc-tagged constitutively-active GTPase mutants	Prof. Laura Machesky, School of Biosciences, The University of Birmingham, UK; Caron & Hall, 1998
pRK5-Rac1-T17N, pRK5-RhoA-T19N, pRK5-Cdc42-T17N	pRK5 constructs containing cDNAs of c-Myc-tagged dominant-negative GTPase mutants	Prof. L. Machesky; Caron & Hall, 1998
pRK5-*sapA*	pRK5 construct containing cDNA of c-Myc-tagged surface array protein	This study
pcDNA3.1; pcDNA3.1-NT-GFP	CMV Promoter, T7 Promoter, MCS, BGH PolyA, SV40 ori, f1 ori, SV40 PolyA, pUC ori, Amp^R, Neo^R; (GFP)	Invitrogen, Karlsruhe, Germany
pcDNA3.1-NT-GFP-Cdc42-T17N	pcDNA3.1-NT-GFP construct containing cDNA of GFP-tagged dominant-negative Cdc42 mutant	Prof. Dr. Ilan Rosenshine, Hebrew University, Tel Aviv, Israel; Ben-Ami *et al.,* 1998
pcDNA3.1-FAK, pcDNA3.1-FAK Y397F, pcDNA3.1-FAK K454R, pcDNA3.1-FAK Pro^-, pcDNA3.1-FAK Y925F	pcDNA3.1 constructs containing cDNAs of HA-tagged wild-type FAK or different FAK mutants	Prof. Christof Hauck, Chair of Cell Biology, University of Konstanz, Konstanz, Germany; Sieg *et al.,* 1999
PDGFRß, DN-PDGFRß	constructs containing cDNAs of wild-type PDGFRß or dominant-negative PDGFRß mutant	Prof. Tony Hunter, The Salk Institute, Molecular Biology and Virology Laboratory, University of California, San Diego, La Jolla, USA
EGFR, DN-EGFR	constructs containing cDNAs of wild-type EGFR or dominant-negative EGFR mutant	Prof. Gordon Gill, Department of Medicine, University of California, San Diego, La Jolla, USA
pEGFP-C2	CMV Promoter, EGFP, MCS, SV40 PolyA, f1 ori SV40 Promoter, SV40 ori, Neo^R, Kan^R HSV TK PolyA, pUC ori	BD Clontech, Heidelberg, Germany
pEGFP-C2-Vav-2, pEGFP-C2-Vav-2 Y172/159F, pEGFP-C2-Vav-2 R425C, pEGFP-C2-Vav-2 W673R and pEGFP-C2-Vav-2 G693R	pEGFP-C2 constructs containing cDNAs of wild-type GFP-tagged Vav-2 or different Vav-2 mutants	Prof. Laszlo Buday, Department of Medical Chemistry, Semmelweis University Medical School, Budapest, Hungary; Tamas *et al.,* 2003
pLP-CMV-Myc	CMV Promoter, SV40 SD/SA, c-Myc, *loxP*, SV40 PolyA, pUC ori, Amp^R,	BD Clontech, Heidelberg, Germany
pLP-CMV-Myc-Vav-2, pLP-CMV-Myc-DN Vav-2	pLP-CMV-Myc constructs containing cDNAs of GFP-tagged wild-type Vav-2 or dominant-negative Vav-2 mutant	Prof. Ch. Hauck; Schmitter *et al.,* 2007
pCR4-TOPO	pUC ori, *lac* Promoter, LacZα-ccdB, Amp^R, Kan^R	Invitrogen
pCR4-TOPO-*sapA*	pCR4-TOPO construct containing cDNA of surface array protein	This study
pGEX-4T-1	pBR322 ori, *lac* I^q, *tac* Promoter, GST, thrombin recognition site, MCS, Amp^R	Amersham Biosciences, Upsala, Sweden
pGEX-4T-1-*sapA*	pGEX-4T-1 construct containing cDNA of surface array protein	This study

4.4. Eukaryotic cells and cell culture conditions

Cells used in this work, listed in Table 5, were grown in indicated media with heat inactivated (56°C, 30 min) fetal bovine serum (FBS) at 37°C in a humidified, 5% CO_2 incubator and were subcultured every two to three days after reaching 90% confluency. Briefly, cells were harvested by trypsin-EDTA treatment, re-suspended in fresh serum-containing medium and dispensed into new culture flask or wells (Greiner Bio-one GmbH, Frickenhausen, Germany).

Table 5. Eukaryotic cells used in this study.

Cell line	Characteristics	Origin/References
Human cell lines		
INT-407	Embryonic intestinal epithelial cells, adherent	ATCC CCL-6
HeLa	Cervix epithelial carcinoma cells, adherent	ATCC CCL-2
THP-1	Leukemic monocytes, suspension	ATCC TIB-202
Mouse cell lines		
GD25	Integrin subunit ß$_1$-deficient fibroblasts, adherent	Prof. Reinhard Fässler, Department of Molecular Medicine, Max Planck Institute of Biochemistry, Martinsried, Germany; Fässler *et al.,* 1995
GD25-ß$_1$A	GD25 stably re-expressing wild-type ß$_1$A	Prof. R. Fässler; Wennerberg *et al.,* 1996
GD25-ß$_1$A$_{TT788-9AA}$	GD25 stably re-expressing mutated integrin subunit ß$_1$A	Prof. R. Fässler; Wennerberg *et al.,* 1998
GD25-ß$_1$A$_{Y783/795F}$	GD25 stably re-expressing mutated integrin subunit ß$_1$A	Prof. R. Fässler; Wennerberg *et al.,* 2000
FAK$^{-/-}$, here called FAK (-)	fibroblasts derived from FAK-deficient mouse embryos	Prof. Ch. Hauck; Sieg *et al.,* 1999
DA2, here called FAK (+)	FAK (-) stably re-expressing HA-epitope-tagged FAK	Prof. Ch. Hauck; Sieg *et al.,* 1999

Cell line	Medium
INT-407	Eagle's Minimum Essential Medium (MEM) containing 2 mM L-glutamine and Earle's salts, 100 units (U)/ml penicillin, 100 µg/ml streptomycin, 10% (FBS) (Invitrogen)
HeLa	Roswell Park Memorial Institute Media (RPMI) 1640 containing 2 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 10% FBS (Invitrogen)
THP-1	RPMI 1640 containing 2 mM L-glutamine, 25mM HEPES, 10% FBS
GD25	Dulbecco's Modified Eagle Medium (D-MEM) containing 4500 mg/L D-glucose, 4 mM L-glutamine, 110 mg/L sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 10% FBS (Invitrogen)
GD25-ß$_1$A, GD25-ß$_1$A$_{TT788-9AA}$, GD25-ß$_1$A$_{Y783/795F}$	See GD25 cells, with 10 µg/ml puromycin
FAK (-)	D-MEM containing 4500 mg/L D-glucose, 4 mM L-glutamine, 110 mg/L sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 10% FBS (Invitrogen)
FAK (+)	See FAK (-) cells, with 200 µg/ml hygromycin

4.5. Infection of host cells with *Campylobacter* strains

For the infection assays, host cells were grown in 6- or 12-well tissue culture plates to reach ~70% confluency. The culture medium was replaced with fresh medium without antibiotics 12 hrs before infection. Bacteria were suspended in phosphate-buffered saline (PBS) (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.137 M NaCl, 2.7 mM KCl, pH 7.4), followed by optical density measurement at λ=600 nm (OD_{600}) in a UV/Vis spectrometer Lambda 2 (Perkin Elmer Waltham, USA). Subsequently, bacteria were added to and co-incubated with host cells at a multiplicity of infection (MOI) of 200 (if not stated otherwise) for the indicated periods of time at 37°C in 5% CO_2.

4.6. Transfection experiments

Transfection is the process in which foreign nucleic acids are introduced into eukaryotic cells. It involves typically opening chemically-created transient pores in the cell plasma membrane, to allow the uptake of DNA material. Transfection is frequently carried out with a positively charged reagent which coats the negative charged DNA, allowing it to fuse with the plasma membrane of eukaryotic cells, releasing the DNA into the cell (Vaheri & Pagano, 1965).
Transient transfections of constructs presented in Table 4 were performed using GeneJammer transfection reagent, according to the manufacturer's instructions (Stratagene, La Jolla, USA). Prior to transfection, the cells were seeded to reach 70-80% confluency. GeneJammer reagent was mixed with DNA, ratio of 3µl/1µg and added to the cells for 48 hrs. The efficiency of transfection was verified by either Western blotting using appropriate antibodies or by immunofluorescence (IF) staining in confocal microscopy.

4.7. RNA interference (RNAi)

RNAi is the process where the introduction of double stranded RNA (dsRNA) into a cell inhibits gene expression in a sequence dependent fashion. RNAi is usually described as a post-transcriptional gene-silencing mechanism in which dsRNA triggers degradation of homologous messenger RNA in the cytoplasm (Hannon, 2002). The mediators of RNA interference are 21- and 22-nucleotide small interfering RNAs (siRNA) which are thought to be generated by the cleavage of longer dsRNA by RNaseIII-type enzymes (Bernstein *et al.,* 2001). In a second step, siRNAs bind to a ribonuclease complex called RNA-induced silencing complex (RISC) that guides the small double stranded RNA to its homologous mRNA target (Hammond *et al.,* 2000). Consequently, RISC cuts the mRNA approximately

in the middle of the region paired with the antisense siRNA, after which the mRNA is further degraded what results in gene silencing (Elbashir *et al.,* 2001; Martinez *et al.,* 2002).

In my studies, siRNA(s) directed against human DOCK180, Vav-2, α-PIX, Tiam1, Trio and control siRNA containing scrambled sequence were purchased from Santa Cruz Biotechnology, Santa Cruz, USA. siRNA(s) targeted against human Rac1, Cdc42 and RhoA were synthesized as follows. Rac1 target sequence (5'-AAAACTTGCCTACTGATCAGT-3'), Cdc42 target sequence (5'-TTCAGCAATGCAGACAATTAA-3'), RhoA target sequence (5'-TACCTTATAGTTACTGTGTAA-3') were utilized. For down-regulation of Tiam1 both the siRNA from Santa Cruz Biotechnology and the one obtained from MWG-Biotech, Ebersberg, Germany [Tiam1 target sequence (5'-ACAGCTTCAGAAGCCTGAC-3')], were used simultaneously.

Transfection of siRNA was performed using siRNA Transfection Reagent according to the manufacturer's instructions (Santa Cruz Biotechnology). Prior to transfection, the cells were seeded to reach 70-80% confluency. siRNA Transfection Reagent was mixed with target or scrambled siRNA at ratio of 5µl/1µg and added to the cells in serum-free medium. After 5 hrs of incubation the normal growth medium containing two-times serum concentration was added without removing the transfection mixture. Cells were assayed 48 hrs post transfection. The efficiency of transfection was verified by Western blotting using appropriate antibodies.

4.8. Gentamicin protection assay

Gentamicin protection assay is a standard method used extensively to analyze cellular invasion by several bacterial species including *Campylobacter* (Kopecko *et al.,* 2001). During infection gentamicin is added, an antibiotic which does not cross the eukaryotic plasma membrane, followed by cell lysis and plating. The bacterial counts obtained from gentamicin-treated cells represent the bacteria internalized and alive whereas the non-protected extracellular bacteria are killed. For these purpose, 4×10^5 cells were seeded in 12-well tissue culture plates and infected as described above. After infection, the cells were washed three times with 1 ml of pre-warmed medium per well to remove non-adherent bacteria. To determine the colony-forming units (CFU) corresponding to intracellular bacteria, the cell monolayers were treated with 250 µg/ml gentamicin (Sigma-Aldrich, Steinheim, Germany) at 37°C for 2 hrs, washed three times with medium, and then incubated with 1 ml of PBS-0.1% (w/v) saponin (Sigma-Aldrich) at 37°C for 15 min. The treated monolayers were re-suspended thoroughly, diluted in BHI medium, and plated on MH agar plates. To determine the total CFU corresponding to host-associated bacteria, the infected monolayers were incubated with saponin without prior treatment with gentamicin. The resulting suspensions

were diluted and plated as described above. For each assay, the level of bacterial adhesion and uptake was determined by calculating the number of CFU. All experiments were routinely performed in triplicates. In parallel control experiments, 250 µg/ml gentamicin killed all extracellular bacteria (data not shown).

4.9. Inhibitor and activator studies

For uptake inhibition and activation studies, host cells in 1 ml medium were pre-treated for 30 min with inhibitors or activators presented in Table 6, followed by infection with *Campylobacter*. In case of Compactin and Toxin B, TcdB, TcdBF cell were pre-treated for 16 hrs and 2 hrs, respectively.

Table 6. Inhibitors and activators used in this study.

Name	Final concentration	Function	Origin/References
Inhibitors			
Compactin	50 µM	Inactivates GTPases by blocking their isoprenylation and membrane targeting	Calbiochem, Darmstadt, Germany; Chong *et al.,* 1994
Toxin B, TcdB toxin	2-6 ng/ml	Mono-glucosylates Rho, Rac and Cdc42, leading to their irreversible inactivation	Dr. Harald Genth, Department of Toxicology, Hannover Medical School, Hannover, Germany; Barbieri & Aktories, 2005; Genth *et al.,* 2006
TcdBF toxin	50 ng/ml	Glucosylates Rac1 and R-Ras but not RhoA and Cdc42, leading to their irreversible inactivation	Dr. Harald Genth, Chaves-Olarte *et al.,* 2003
cell-permeable C3 toxin (C2IN-C3 together with C2II)	1 µg/ml	ADP-Ribosylates RhoA-C leading to their inactivation	Dr. Harald Genth, Genth *et al.,* 2003
NSC23766	50 µM	Inhibits Rac1 GDP/GTP exchange activity by interfering with the interaction between Rac1 and its GEFs Tiam1 and Trio	Calbiochem; Gao *et al.,* 2004
Genistein	250 µM	Inhibits broad-spectrum of tyrosine kinases	Calbiochem; Wooldridge *et al.,* 1996; Biswas *et al.,* 2004; Hu *et al.,* 2006a
Tyrphostin-46	600 µM	Inhibits EGFR, $p56^{Lck}$ and PDGFR	Biomol, Hamburg, Germany; Biswas *et al.,* 2004
Wortmannin	1 µM	Inhibits PI3 kinase	Calbiochem; Biswas *et al.,* 2000; Hu *et al.,* 2006a
Staurosporine	0.4 µM	Inhibits broad-spectrum of serine/threonine kinases	Calbiochem; Biswas *et al.,* 2004
PP2	50 µM	Inhibits Src tyrosine kinase	Calbiochem; Hanke *et al.,* 1996

See next page for continuation of table.

Table 6. Inhibitors and activators used in this study, continued

Name	Final concentration	Function	Origin/References
Activators			
CNF-1	350 ng/ml	Deamidates and trans-glutaminates Rho, Rac, and Cdc42, leading to their activation	Prof. Gudula Schmidt; Institute for Experimental and Clinical Pharmacology and Toxicology, Albert Ludwigs University Freiburg, Freiburg, Germany; Schmidt *et al.,* 1997
CNF-1 (C866S)	350 ng/ml	Functionally inactive CNF-1 carrying a single point mutation (C866S)	Prof. G. Schmidt; Schmidt *et al.,* 1998
CNF-Y	350 ng/ml	Deamidates and specifically activates RhoA	Prof. G. Schmidt; Hoffmann *et al.,* 2004; Hoffmann & Schmidt, 2004

4.10. GTPase activation assays

Rac1 and Cdc42 activation in infected cells was determined with the Rac1 and Cdc42 activation assay kit (Cytoskeleton, Denver, USA). The assay uses the Cdc42/Rac1 Interactive Binding (CRIB) region (also called the p21 Binding Domain, PBD) of p21 activated kinase 1 (PAK), an Rac1/Cdc42 effector protein (Benard *et al.,* 1999). The PBD protein motif has been shown to bind specifically to the active, GTP-bound form of Rac1 and/or Cdc42 proteins (Burbelo *et al.,* 1995; Zhao & Manser, 2005). The PAK-PBD is in the form of a GST fusion protein, which allows one to "pull-down" the PAK-PBD/GTP-Rac1 (or GTP-Cdc42) complex with glutathione affinity beads. The assay therefore provides a simple method for quantifying Rac1 or Cdc42 activation in cells.

Host cells were grown to 80% confluency in 175 cm^2 tissue culture flasks (Greiner Bio-one GmbH) and serum-starved overnight. Subsequently, cells were incubated in culture medium, as a control, or infected with *C. jejuni* suspended in medium (MOI of 200) for indicated periods of time. Uninfected and infected cells were washed with PBS, re-suspended in the lysis buffer of the kit, and detached from dishes with a cell scraper. The lysates were centrifuged (5000 x *g*, 5 min, 4°C) and the total protein concentration in each lysate was determined by BCA assay (Pierce, Rockford, USA). Two mg of total protein for each activation assay reaction was used. For a positive and negative control, the GTPγ-S and GDP were added to uninfected cell lysates for 15 min, respectively. Cell lysates (treated with bacteria, GTPγ-S, GDP or untreated) were mixed with the PAK-RBD slurry and incubated at 4°C on a rotator for 1 h. Finally, the beads were collected by centrifuging (5000 x *g*, 3 min, 4°C), washed two times with assay buffer and re-suspended in 15 µl of

SDS buffer. Activated Rac1 and Cdc42 were then visualized by immunoblotting. To confirm equal amounts of protein for each sample, aliquots of the lysates from different time points were also analyzed by immunoblotting. The GTPase activities were quantified as band intensities representing the amount of active Rac1-GTP and Cdc42-GTP using the Lumi-Imager F1 software program (Roche, Mannheim, Germany).

4.11. Immunoprecipitation

Immunoprecipitation (IP) is the technique of precipitating an antigen out of a given suspension using an antibody specific to that antigen. This process can be used to separate and enrich a specific protein from whole cell lysates or culture supernatants. Co-immunoprecipitation ("pull-down") can identify protein complexes present in cell extracts; by immunoprecipitating one protein known to be in a complex, additional members of the complex can also be identified. The complexes are enriched using insoluble antibody-binding proteins such as Protein A and Protein G coupled to sepharose beads. After multiple washing steps with PBS, the precipitate can be analyzed by Western blotting.

In typical experiments, 1 x 10^7 INT-407 or THP-1 cells were washed with cold PBS and lysed for 30 min at 4°C in lysis buffer [20 mM Tris pH 7.2, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM Na_3VO_4, protease inhibitor cocktail (Roche)]. To lower the amount of non-specifically-attached proteins, lysates were pre-cleared by incubation with protein G-Sepharose (Amersham Biosciences) for 2 hrs at 4°C. Subsequently, the lysates were centrifuged (3000 x *g*, 5 min, 4°C), and then 2 µg of α-SapA or α-Tiam1 (Santa Cruz Biotechnology) antibodies were added to the supernatants and incubated overnight at 4°C. Immune complexes were precipitated by the addition of protein G-Sepharose for 2 hrs, washed once with lysis buffer and tree times with 0.5 x PBS and finally re-suspended in 80 µl of SDS buffer. Samples were analyzed by SDS-PAGE and immunoblotting.

4.12. Determination of protein concentration

The protein concentration was quantified with BCA Protein Assay (Pierce). The assay is based on reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) and colorimetric detection of the cuprous cation (Cu^{+1}) using reagent containing bicinchoninic acid (BCA) (Smith *et al.,* 1985). The reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This complex exhibits a strong absorbance at λ=562 nm.

For this purpose, protein samples were diluted, mixed with BCA working reagent and incubated for 30 min at 60°C. Subsequently, the absorbance of the samples was

measured at λ=562 nm. In parallel series of dilutions of known concentration (5-250 µg/ml) of bovine albumin serum (BSA) (Pierce) were prepared and assayed alongside. The concentration of proteins was determined based on standard curve obtained from BSA measurement.

4.13. Generation of the CadF and SapA antibodies

Polyclonal antiserum α-CadF-1 was raised according to standard protocols (BioGenes, Berlin, Germany) by immunization of two rabbits with a conserved CadF-derived peptide (amino acids 293-306: QDNPRSSNDTKEGR) conjugated to Limulus polyphemus haemocyanin carrier protein. The polyclonal rabbit antiserum α-CadF-2 was gained by immunization with gel-purified CadF protein (Konkel *et al.,* 1997) and was kind gift of Prof. Michael Konkel (School of Molecular Biosciences, Center for Biotechnology, Washington State University, Pullman, USA). Antiserum α-SapA was obtained by immunization of mice with 470 µg of purified SapA-GST (1.3 mg/ml in PBS pH 7.4) conjugated to Limulus polyphemus haemocyanin carrier protein (BioGenes).

4.14. SDS polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

SDS-PAGE involves the separation of proteins based on their size. By heating the sample under denaturing and reducing conditions, proteins become unfolded and coated with SDS detergent molecules, acquiring a high net negative charge that is proportional to the length of the polypeptide chain. When loaded onto a gel matrix and placed in an electric field, the negatively charged protein molecules migrate towards the positively charged electrode and are separated by a molecular sieving effect. Following electrophoresis, proteins in a polyacrylamide gel can be visualized by staining (*e.g.* Coomassie staining) or blotted onto a positively charged membrane and probed with protein-specific antibodies. With the semi-dry electro-blotting method, the gel and membrane are sandwiched between two stacks of filter paper that have been pre-wet with transfer buffer. The membrane is placed near the anode, and the gel is placed near the cathode. Proteins are transferred to the membrane when an electric current is applied. The specificity of the antibody-antigen interaction enables a single protein to be identified in the midst of a complex protein mixture that has been immobilized on a membrane. The membrane is blocked to prevent any non-specific binding of antibodies to its surface and then primary antibody is added. In order to locate it, a secondary antibody is applied which binds to all IgG antibodies from animal species in which primary antibody was generated. The secondary antibody is chemically coupled to

a reporter, *e.g.* to an enzyme that after addition of appropriate substrate produces luminescent reaction products which allows its detection.

Whole bacterial cells harvested from agar plates or proteins from transfected and/or infected cells were lysed in SDS-PAGE buffer (2% SDS, 10 % glycerol, 0.01% bromophenol blue, 62.6 mM Tris-HCl pH 6.8, 0.1 M DTT) (Fermentas, St. Leon-Rot, Germany), boiled at 95°C for 5 min, separated on 6-15 % polyacrylamide gels (depending on the size of the protein of interest), and either stained with Coomassie-Brilliant Blue or blotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore, Bedford, USA). As a standard, PageRuler Prestained Protein Ladder Plus (Fermentas) was used. Preparation, running, blotting and staining of the gels was performed according to Sambrook *et al.,* 1989 with use of Mini-Protean 3 gel system, (120 V, 1.5-2 hrs, RT) (Bio-Rad Laboratories, Hercules, USA), semi-dry blotting apparatus (0.8 mA/cm^2, 2 hrs, RT) (Roth, Karlsruhe, Germany) and buffers listed below.

Gel running buffer pH 8.3	
per liter	
Tris	3.2 g
Glicine	18.8 g
10% (v/v) SDS	10 ml

Transfer buffer pH 8.4	
per liter	
Tris	3.0 g
Glicine	14.5 g
10% (v/v) SDS	10 ml
Methanol	200 ml

10xTBS buffer pH 7.4	
per liter	
Tris	24.2 g
NaCl	80.0 g

TBS-T buffer pH 7.4	
per liter	
10xTBS pH 7.4	100 ml
Tween 20	1ml

Coomassie stain	
per liter	
Coomassie Briliant Blue	2.5 g
Methanol	450 ml
Acetic acid	100 ml

Coomassie de-stain sol.	
per liter	
Methanol	300 ml
Acetic acid	100 ml

After blotting, membranes were blocked with TBS-T-5%-BSA or TBS-T-5% non-fat dry milk, either at 4°C overnight or 1-2 hrs at RT. Subsequently, membranes were incubated with primary antibodies, listed in Table 7, overnight at 4°C or 2 hrs at RT rotating, according to the manufacturer's instructions and then washed three times for 10 min with TBS-T. As secondary antibodies, horseradish peroxidase-conjugated α-mouse IgG, α-rabbit IgG or α-goat IgG were applied for 1 h at RT, rotating (DakoCytomation, Hamburg, Germany), followed by washing three times for 15 min with TBS-T. Immuno-reactive bands were visualized by ECL plus Western Blotting Detection System (Amersham Biosciences).

Table 7. Antibodies used in this study for Western Blotting analysis.

Antibody [a, b]	Origin [a, b]	Purchased from
Monoclonal		
α-GFP (JL-8)	mouse	BD Biosciences
α-c-Myc antibody (hybridoma clone 9E10)	mouse	ATCC, CRL-1729
α-Rac (clone 23A8)	mouse	Upstate Biotechnology, USA
α-Rho A (26C4)	mouse	Santa Cruz Biotechnology
α-HA (6E2)	mouse	Cell Signaling, Danvers, USA
α-DOCK180 (H-4)	mouse	Santa Cruz Biotechnology
α-Tyr (PY99)	mouse	Santa Cruz Biotechnology
Polyclonal		
α-CadF-1	rabbit	This study
α-CadF-2	rabbit	Konkel *et al.,* 1997
α-GAPDH (V-18)	goat	Santa Cruz Biotechnology
α-Cdc42 (P1)	rabbit	Santa Cruz Biotechnology
α-Integrin ß1	rabbit	Cell Signaling
α-FAK (A17)	rabbit	Santa Cruz Biotechnology
α-FAK-PY-397	rabbit	Biomol, Hamburg, Germany
α-Vav-2 (H-200)	rabbit	Santa Cruz Biotechnology
α-PIX (Q-20)	goat	Santa Cruz Biotechnology
α-Tiam1 (C-16)	rabbit	Santa Cruz Biotechnology
α-Trio (D-20)	goat	Santa Cruz Biotechnology
α-EGFR	rabbit	Cell Signaling
α-PDGFR ß	rabbit	Cell Signaling
α-SapA	mouse	This study

To re-probe the membrane with another primary antibody, membrane was incubated in stripping buffer for 45 min at 60°C, washed extensively in TBS-T, blocked and probed again as described above.

Stripping buffer pH 6.7	
per 100 ml	
1M Tris	6.25 ml
10% (v/v) SDS	20.0 ml
ß-merkaptoethanol	0.833 ml

4.15. Enzyme-linked immunosorbent assay (ELISA)

ELISA is a method that is used for the quantitative assay of proteins in solution. In sandwich ELISA, proteins are immobilized on a solid support (*e.g.* the wells of a 96-well plate) and used as capture molecules to bind the protein that is being assayed. After a wash step to remove non-specifically bound material, a secondary antibody, specific for the target protein,

is added. This secondary antibody is usually conjugated to an enzyme that allows its detection by chromogenic or chemiluminescent methods. In parallel series of dilutions of known concentration of a standard are assayed alongside. The concentration of protein is determined based on standard curve obtained from standard measurement.

In my studies, the amount of IL-8 secreted into the cell culture supernatants was determined by ELISA using the OptEIA™ human IL-8 kit (BD Biosciences) and Quantikine mouse KC kit (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany), according to the manufacturers' instructions. For this purpose, 96-well plates were coated with 100 µl/well of a human IL-8 monoclonal antibody diluted (1:250) in Coating Buffer (0.1 $NaCO_3$ pH 9.5) and incubate overnight at 4°C. Wells were aspirated and washed three times with 300 µl/well of Wash Buffer (PBS-0.05% Tween 20). Plates were blocked with 200 µl/well of Assay Diluent (PBS-10% FBS) and incubated for 1 h at RT. Wells were aspirated and washed three times with 300 µl/well of Wash Buffer. Standard and sample dilutions (1:20) in Assay Diluent were prepared. 100 µl of standard or sample was pipetted into appropriate wells and incubated for 2 hrs at RT. Wells were aspirated and washed three times with 300 µl/well of Wash Buffer. 100 µl of Working Detector (biotinylated anti-human IL-8 monoclonal antibody, streptavidin-horseradish peroxidase conjugate reagent) was added to each well and incubated for 1 h at RT. Wells were aspirated and washed three times with 300 µl/well of Wash Buffer. 100 µl of Substrate Solution (tetramethylbenzidine (TMB) and hydrogen peroxide) was added to each well and incubate for 30 min at RT in the dark. The reaction was stopped by adding of 50 µl of Stop Solution (2 N H_2SO_4) to each well. The absorbance was measured at λ=450 nm with λ correction at 570 nm in a microplate reader (Spectrafluor Plus, TECAN, Crailsheim, Germany). Values were calculated from a standard curve. IL-8 secreted into the mouse fibroblast cell culture supernatants was quantified according to procedure described above. The polyclonal antibody specific for mouse KC (functional IL-8 homologue) (Lee *et al.,* 1995) and horseradish peroxide-linked polyclonal antibody specific for mouse KC were used as capture and secondary antibody, respectively. As Stop Solution hydrochloric acid was applied.

4.16. Isolation of DNA

Plasmid DNA isolation from *E. coli* was carried out with the modified alkaline/SDS lysis (Birnboim & Doly, 1979) and anion exchange adsorption method using JETSTAR plasmid purification system (Genomed, Löhne, Germany), according to the manufacturer's instructions. For this purpose, *E. coli* cells were prepared by alkaline/SDS lysis and after neutralization, applied onto JETSTAR Columns (Mini, Midi or Maxi). The plasmid DNA, selectively bound to the anion exchange resin, was washed to remove impurities. Finally,

the purified plasmid DNA was eluted from the column and concentrated by isopropanol precipitation. The precipitated DNA was re-dissolved in water.

The presence or absence of *Campylobacter* plasmids such as pVir (Bacon *et al.,* 2000; 2002) was evaluated by standard plasmid isolation as depicted above.

Genomic DNA was extracted as described by Wilson, 1989. *Campylobacter* growing on *Campylobacter* selective agar plate were suspended in PBS and harvested by centrifuging (5000 x *g*, 5 min, RT). The pellet was mixed with 200 µl of lysis buffer (50 mM EDTA, pH 8.0, 1 % SDS, 0.1 mg/ml proteinase K) and kept at 55°C for 1-2 hrs to ensure complete cell lysis. Subsequently, one-tenth volume of 3 M sodium acetate pH 5.5 was added. To remove proteins, solution was extracted with a mixture of phenol: chloroform: isoamyl alcohol (25:24:1) and then with chloroform to get pure DNA. The DNA-containing aqueous phase was separated by centrifuging (13000 x *g*, 10 min, RT). DNA was precipitated with 2.5 volumes of absolute ethanol and collected by centrifuging (13000 x *g*, 30 min, 4°C). Co-precipitated salts were removed by washing with 70% (v/v) ethanol. DNA was dried at RT and re-suspended in 50 µl of water. The purified DNA was used as template for PCR amplification.

4.16.1. Determination of DNA concentration and quality

DNA concentration and quality was determined spectro-photometrically with use of Biophotometer and UVette cuvettes (Eppendorf, Hamburg, Germany), according to Sambrook *et al.,* 1989. The concentration of DNA in aqueous solutions can be estimated by adjusting the A_{260} measurement for turbidity (measured by absorbance at A_{320}), multiplying by the dilution factor, and using the relationship that an A_{260} of 1.0 = 50 µg/ml pure DNA.

DNA concentration (µg/ml) = (A_{260} reading – A_{320} reading) × dilution factor × 50 µg/ml

By additional measurement at λ=280 nm, the purity of DNA can be estimated. Samples with A_{260}/A_{280} ratio between 1.7 and 2.0 were accepted as high-quality DNA and were used for transfection experiments.

4.17. Restriction, modification and purification of DNA

4.17.1. Cleavage of DNA with restriction enzymes

Restriction enzymes were used according to the manufacturer's instructions (New England Biolabs GmbH, Frankfurt am Main, Germany). For analytic restriction, 20 µl reaction mixes containing: 0.5 µg of DNA and 5 U of enzyme were prepared. For preparative restriction,

30 µl reaction mixes containing 2.5 µg of DNA and 15 U of enzyme were used. Reaction mixes were incubated 2 hrs at the optimal temperature for the chosen enzymes.

4.17.2. DNA Ligation

Ligations were carried out using the Rapid DNA Ligation Kit (Fermentas) according to the manufacturer's instructions.
The molar ratio of insert DNA to vector DNA used was 3:1. The amount of applied insert DNA was calculated using the following formula:

$$\frac{\text{X ng vector x Y kb insert}}{\text{Z kb vector}} \quad \frac{3}{1} = \text{N ng insert}$$

X, amount of vector DNA
Y, length of insert DNA
Z, length of vector DNA
N, amount of applied insert DNA

For cloning of PCR-amplified *sapA,* TOPO TA Cloning Kit with topoisomerase I covalently bound to the pCR4-TOPO vector (Invitrogen) was used, according to the manufacturer's instructions. Subsequently, ligation preparation was transformed into *E. coli* competent cells (see 4.20.)

4.17.3. Agarose gel electrophoresis

Agarose gel electrophoresis was used for separation and size determination of DNA fragments. Agarose gels 0.8% (w/v) with 0.1 µg/ml ethidium bromide and 0.5 x TBE-buffer (44.5 mM Tris, 44.5 mM Boric acid, 1 mM EDTA, pH 8.0), as running buffer were utilized. Before application on the gel, samples were mixed with 1/5 volume of 6x loading buffer (0.09% (w/v) bromophenol blue, 0.09% (w/v) xylene cyanol, 60% (v/v) glycerol, 60 mM EDTA) (Fermentas). For determination of DNA fragment sizes, GeneRuler 1 kb DNA Ladder (Fermentas) was applied. Ultraviolet-induced fluorescence, emitted by ethidium bromide molecules intercalated into DNA, was detected with use of the Lumi-Imager F1 (Roche).

4.17.4. Purification of DNA fragments and extraction from agarose gel

Purification of DNA fragments was performed by agarose gel electrophoresis followed by gel extraction using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. DNA bands of interest were excised from the 0.8% (w/v) agarose gels stained with 0.04% (w/v) methylene blue and solubilized by addition of three volumes of QG buffer and incubation at 50°C for 10 min. Subsequently, sample was applied to the QIAquick column. DNA selectively bound to the resin was washed with PE buffer and eluted with 30 µl of water. Extracted DNA was verified by standard agarose gel electrophoresis.

4.18. Polymerase chain reaction (PCR)

PCR was used for amplification of DNA fragments, cloning and verification of bacterial strains. Reactions were carried out in the PTC-225 Peltier Thermal Cycler (MJ Research, Waltham, USA). DNA was amplified with pairs of specific primers (MWG-Biotech, Ebersberg, Germany) and Taq DNA polymerase (Qiagen) in PCR reaction mix as listed below. *Pfu* DNA Polymerase (Fermentas) was used for high fidelity amplification. The PCR program included: one denaturation step (94°C, 5 min) and 35 times repeated cycles consisting of denaturation, oligonucleotide hybridization (primer annealing) and DNA synthesis. The annealing temperature used was 4°C lower than the T_m value of the shorter primer. The T_m value of primers depends on oligonucleotides' base composition and can be calculated with the following formula: $T_m = 2*\Sigma(AT) + 4*\Sigma(GC)$. The elongation times used were depending on the length of DNA fragments which were to be amplified and were calculated as follows: 1 min per 1000 bp.

Reagents	Final concentration
dNTP-Mix	0,2 mM
Each primer	100 pmol
DNA (template)	50 ng
10 x PCR buffer	5 µl
with $MgCl_2$	3 mM
Taq-polymerase	2,5 U
H_2O	Until 50µl

4.18.1. PCR Primers

Primers used for amplification and cloning of the surface array protein gene *sapA*:

SapA Fwd: 5'-CG**GGATCC**ATGTTAAACAAAACAGATGTTTCAATG-3'
SapA Rev: 5'-G**GAATTC**TTAAATTACGCTTCCATCATCAAC-3'

Bold, *Bam*HI restriction site (SapA Fwd) and *Eco*RI restriction site (SapA Rev). Underlined, start and stop codon, respectively. SapA Fwd and SapA Rev correspond to positions: 43863-43889 and 46682-46659 in the *C. fetus* strain 23D *sap* gene locus sequence AY211269, NCBI.
Primers used by cooperation partner Lieke B. van Alphen (Department of Infectious Diseases and Immunology, Utrecht University, The Netherlands) for amplification, cloning and sequencing of CadF protein gene *cadF* and its flanking regions:

CadF1 Fwd: 5'-TTGCTCTAAAGGATAACCTATGA-3'
CadF1 Rev: 5'-TATGGACGCCGCAAAGCAAG-3'
CadF2 Fwd: 5'-CCACTCTTCTATTATCCGCTCTACC-3'
CadF2 Rev: 5'-GGTGCTGATAACAATGTAAAATTTG-3'

Amplified products were analyzed by agarose gel electrophoresis, cloned into pGEM-T-easy vector (Promega, Madison, USA) and sequenced by L. van Alphen.

4.19. Preparation of *E. coli* chemically competent cells

For preparation of *E. coli* chemically competent cells, 10 ml LB medium was inoculated with a single colony of TOP10 or BL21 (Invitrogen) from a fresh plate and incubated overnight at 37°C, 200 rpm. Subsequently, 100 ml LB medium was inoculated with 1 ml of this pre-culture and grown to an OD_{600} of 0.45-0.55. The culture was chilled on ice and centrifuged (4000 x *g,* 10 min, 4°C). The pellet was re-suspended in 50 ml of cold 0.1 M $CaCl_2$ and incubated on ice for 30 min. Cells were centrifuged again and re-suspended in 5 ml of cold 0.1 M CaCl2/10% (v/v) glycerol. 100 µl aliquots were shock frozen on dry ice and stored at -80°C.

4.20. Transformation of chemically competent *E. coli*

Transformation was performed according to standard protocols (Hanahan *et al.,* 1983). For this purpose, 100 µl of chemically competent *E. coli* TOP10 or BL21 cells were thawed on ice and mixed with 0.25 µg plasmid DNA or the total ligation sample. After 15 min on ice, cells were heat-shocked for 30 s at 42°C for DNA incorporation. Afterwards, 1 ml of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added and cells were regenerated for 45 min at 37°C under shaking at 200 rpm and plated on the corresponding LB selective plates.

4.21. Overexpression and purification of *C. fetus* surface array protein SapA

Fresh overnight culture of BL21-pGEX-4T-1-*sapA* was inoculated 1:100 in 2xYT medium supplemented with 100 µg/ml ampicillin and grown at 37ºC with shaking at 200 rpm to an OD_{600} of 0.75. Overexpression was induced in the presence of 1 mM IPTG at 30ºC for 4 hrs shaking at 200 rpm. After induction of SapA, the bacterial culture was centrifuged (4000 x *g*, 15 min, 4ºC). The pellet obtained was stored at -80ºC until further processing. Whole cell lysate was prepared as follows: bacterial pellet was re-suspended in B-PER Reagent (Pierce) in 1/25 of the original culture volume. Protease inhibitor cocktail (Roche) was added to the pellet resuspension immediately. Once a homogenous mixture was established it was gently shaken at RT for 10 min. Whole cell lysate obtained was then centrifuged in rotor JA 25.5 in a Beckman Avanti J-25 centrifuge (Beckman Coulter, Fullerton, USA) (27000 x *g*,15 min, 4ºC) to separate soluble from insoluble proteins. Supernatant containing soluble proteins was incubated with 10 ml of glutathione S-transferase (GST) immobilized resin (Pierce) pre-equilibrated with B-PER Reagent. Incubation was carried out at RT with gentle shaking for 1 h, after which the slurry was transferred to a low-pressure chromatography column and the breakthrough was collected. The resin was than washed with nine column volumes of Wash Buffer 1 (Pierce) followed by three column volumes of Wash Buffer 2 (Pierce). Elution was carried out with six column volumes of 5 mM reduced glutathione in Wash Buffer 2 (Pierce). Eluate was collected in 4-ml fractions. SapA eluted as a predominant species in the second (main) and third fraction (side fraction). Each of the fractions was then concentrated four-fold with use of Amicon Ultra centifugal filter devices (Millipore) before final step of purification by gel filtration through a HiLoad 16/60 Sephacryl S-200 HR gel filtration column (Amersham Biosciences) in PBS pH 7.4. Gel filtration was carried out at 4ºC at a flow rate of 1 ml/min using an AKTAprime FPLC pump system (Amersham Biosciences). The protein concentration of both fractions obtained was determined by BCA protein assay (Pierce). For cleavage of SapA from GST, 5-10 U of

thrombin per 1 mg of fusion protein was used according to the manufacturer's instructions (Amersham Biosciences). During cleavage performed at RT or 37°C, samples were removed at various time points (1-24 hrs) and subjected to SDS-PAGE/Coomassie staining analysis, to estimate extent of digestion, which even after many optimizing trials, was weak. GST was purified according to procedure described above with the following exceptions: culture of BL21-pGEX-4T-1 was grown to an OD_{600} of 0.78. Overexpression was induced in the presence of 1 mM IPTG at 37ºC for 2 hrs. As first purification step yielded pure GST the gel filtration was omitted.

4.22. *In vitro* SapA phosphorylation assay with recombinant c-Src or c-Abl

To prove whether SapA can be phosphorylated by host cell tyrosine kinases, *in vitro* kinase assays using recombinant c-Src kinase or c-Abl kinase were performed. For this purpose, 10^{10} *C. fetus* cells were harvested and lysed in 1 ml ice-cold kinase buffer (25 mM HEPES pH 7.0, 150 mM NaCl, 10 mM $MgCl_2$, 1% Nonidet P-40, 5 mM dithiothreitol (DDT), 1 mM Na_3VO_4, protease inhibitor cocktail (Roche) (Selbach *et al.,* 2002). Five U of recombinant human c-Src (Upstate, USA) and 1 µM adenosine-5'-tri-phosphate (ATP) were mixed with 30 µl of the *C. fetus* lysate and incubated for 30 min at 30°C. In a similar experiment, 1 µg of purified SapA-GST or SapA was mixed with 25 µl of ice-cold kinase buffer and incubated with 1.5 U of recombinant human c-Src (Upstate) or c-Abl (New England Biolabs) and 1 µM ATP for 30 min at 30°C. Reactions were stopped by addition of SDS. Samples were analyzed by SDS-PAGE and immunoblotting.

4.23. Preparation of SapA-coated latex beads

Latex beads (1.1 µm in diameter, Sigma-Aldrich) were coated with SapA protein as follows: the SapA-GST was dialyzed with use of Slide-A-Lyzer Mini Dialysis Units (Pierce) against coupling buffer (50 mM MES pH 6.1, 200 mM NaCl) for 12 hrs at 4°C. The beads were incubated with SapA-GST protein (0.5 mg/ml) or with GST (0.5 mg/ml) as negative control in coupling buffer at 4°C overnight. Beads were then washed three times with coupling buffer and nonspecific sites were blocked with 2% BSA at 37°C for 1 h. After washing with PBS-0.1% BSA, the SapA-coated beads were finally re-suspended in PBS-0.02% BSA.

4.24. Analysis of the binding and internalization of SapA-coated latex beads into host cells

Cells were grown in MEM/D-MEM with 10% FBS on poly-L-lysine-coated glass coverslips at 37°C in 5% CO_2 to 70-80% confluence. Cell monolayers were co-incubated with protein-

coated latex beads at an approximate cell:bead ratio of 1:200 (binding studies) and 1:100 (internalization studies) at 37°C in 5% CO_2 for indicated periods of time. After gentle washing with medium, cells were fixed with 3.8% (v/v) paraformaldehyde (PFA) and subjected to phase contrast microscopy or double IF staining as described below. The number of extracellular and intracellular beads was counted in 80 randomly selected cells, based on micrographs obtained after double IF staining. For binding experiment, 25 randomly selected cells were analyzed.

4.25. Cell attachment assay

Cell attachment assays were used to investigate cell binding on ligand-coated wells. Bound cells were quantified by crystal violet staining and subsequent absorbance measurement (Steffensen *et al.,* 1998). For this purpose, 96-well MICROLON ELISA plates (Greiner Bio-one GmbH) were coated with SapA-GST protein (50 µg/ml in 100 µl PBS/well) at 4°C overnight. Positive control wells were coated with 50 µg/ml fibronectin (Chemicon International, USA) whereas (50 µg/ml) GST and BSA served as negative controls. Nonspecific binding sites were blocked by incubation with heat-denatured PBS-5% BSA for 2 hrs at RT. After washing with PBS, 4 x 10^4 INT-407 cells in serum-free MEM were allowed to attach to wells for 4 hrs at 37°C in humidified atmosphere supplemented with 5% CO_2. Cells were then washed with MEM and fixed with 3.8% (v/v) PFA for 15 min at RT. Adherence of INT-407 cells was firstly assessed by phase contrast microscopy (LH50A, Olympus, Tokyo, Japan), then the bound cells were stained with 0.5% crystal violet in 20% methanol for 15 min at RT. After extensive rinses in PBS, cellular stain was dissolved in 10% acetic acid, and cell numbers were quantified by measurement of the absorbance at λ=590 nm in a microplate reader (Spectrafluor Plus, TECAN).

4.26. Immunofluorescence labeling, Fluorescence and Confocal Laser Scanning Microscopy (CLSM)

Specimens were fixed in 3.8% (v/v) PFA at RT for 15 min followed by permeabilization of the mammalian cell membrane with PBS-0.1% Triton X-100 for 15 min. All antibodies were diluted in PBS-1% BSA, and all incubations with antibodies were carried out for 1 h. Filamentous actin in the host cell was labeled with rhodamine- or fluorescein isothiocyanate (FITC)-conjugated phalloidin (Molecular Probes, Eugene, Oregon, USA; Sigma-Aldrich, respectively). Expression of GTPase constructs was detected using an α-c-Myc antibody, followed by Alexa 350-labeled α-mouse IgG (Molecular Probes).

For differential staining of intracellular and extracellular SapA-coated latex beads, fixed cells were probed with mouse α-SapA antibody followed by Alexa 350-labeled α-mouse IgG (Molecular Probes). Subsequently specimens were permeabilized with PBS-0.1% Triton X-100 for 15 min and probed with α-SapA antibody, followed by tetramethylrhodamine isothiocyanate (TRITC)-labeled α-mouse IgG (Sigma-Aldrich). IF-labeled samples were analyzed in cooperation with Dr. Roland Hartig, Otto von Guericke University, Magdeburg, Germany, with a Leica DMRE7 fluorescence microscope and TCS SP2 confocal laser scanning microscope (Leica Microsystems, Bensheim, Germany). Confocal image data obtained with a 100×/1.4 N.A. oil immersion objective and CLSM-software (Leica Microsystems) were processed digitally using Velocity software (version 3.6) which allows optimizing images in brightness and contrast and pseudo-coloring: GFP, FITC (green), rhodamine, TRITC (red) and Alexa-350 (blue) and the determination whether the bacteria are inside or outside of infected cells.

4.27. Field Emission Scanning Electron Microscopy (FESEM)

Cells grown on coverslips were infected with *C. jejuni* for either 4 hrs or 6 hrs, then fixed with a fixation solution containing 5% formaldehyde and 2% glutaraldehyde in cacodylate buffer (0.1 M cacodylate, 0.01 M $CaCl_2$, 0.01 M $MgCl_2$, 0.09 M sucrose; pH 6.9) and subsequently washed several times with cacodylate buffer. Samples were further processed by cooperation partner Dr. Manfred Rohde (Helmholtz Center for Infection Research, Braunschweig, Germany). Briefly, fixed samples were dehydrated with a graded series of acetone (10, 30, 50, 70, 90 and 100%) on ice for 15 min for each step. Samples in the 100% acetone step were allowed to reach RT before another change of 100% acetone. They were then subjected to critical-point drying with liquid CO_2 (CPD030, Balzers, Liechtenstein). Dried samples were covered with a 10 nm thick gold film by sputter coating (SCD040, Balzers Union, Liechtenstein) before examination in a field emission scanning electron microscope (Zeiss DSM-982-Gemini) using the Everhart Thornley SE detector and the inlens detector in a 50:50 ratio at an acceleration voltage of 5 kV.

4.28. Matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS)

Mass spectrometry is an analytical method that determines the molecular mass of a sample. Once inside the ionization source, the sample molecules are ionized. The ions are extracted into the analyzer region of the mass spectrometer where they are separated according to their mass (m) -to-charge (z) ratios (m/z). The separated ions are detected and this signal

sent to a data system where the m/z ratios are stored together with their relative abundance for presentation in the format of a m/z spectrum, which can be used *e.g.* to generate protein sequence information.

In-Gel Digestion, Peptide Extraction and MALDI-MS were performed by cooperation partners Dr. Manfred Nimtz (Helmholtz Center for Infection Research, Braunschweig, Germany) and Dr. Sophie Haebel (Interdisciplinary Research Center for Mass Spectrometry of Biopolymers, University of Potsdam, Germany).

Protein bands were excised from Coomassie-stained SDS-PAGE gel and washed two times for 15 min with 50 mM ammonium bicarbonate, pH 8.0 and two times for 15 min with 60% acetonitrile/0.5% formic acid. After drying under vacuum, protein spots were reduced with 10 mM DTT for 20 min at 56°C and alkylated with 100 mM iodoacetamide for 20 min at RT in the dark. Then gel spots were washed with 50 mM ammonium bicarbonate, pH 8.0, followed by 60% acetonitrile/ 0.5% formic acid and dried as described above. Protein digestion was performed with trypsin (Promega) according to the manufacturer's instructions. For peptide extraction, gel pieces were washed with 50 mM ammonium bicarbonate and 60% acetonitrile/0.5% formic acid as described above. The extracts were pooled and concentrated under vacuum to a final volume of 20 µl. ZipTip C18 Sepharose tips (Millipore) were used to desalt and concentrate the peptide samples to a volume of 10 µl. Proteolytic peptides were analyzed with a Bruker ULTRAFLEX time-of-flight (TOF/TOF) instrument using a matrix of 19 mg α-cyano-4-hydroxy-cinnamic acid in 400 µl of acetonitrile and 600 µl of 0.1% (v/v) trifluoroacetic acid in H_2O. Samples of 1 µl and an approximate concentration of 1-10 pmol/µl were mixed with equal amounts of matrix. This mixture was spotted onto a stainless steel target and dried at RT before analysis. Matrix Science (http://www.matrixscience.com) and NCBI Data Bases (http://www.ncbi.nlm.nih.gov) were used for evaluation of the generated spectra.

4.29. Statistical analysis and database search

All data were analyzed using the Student's t-test with SigmaStat statistical software (version 2.0). $P<0.05$ were considered as statistically significant. Data are shown as mean values ± standard deviation. Nucleotide sequence analysis and protein sequence alignments were performed using free software (http://searchlauncher.bcm.tmc.edu/seq-util/Options/sixframe.html; http://www.ebi. ac.uk/clustalw) and NCBI, ExPASy Data Bases.

Unless not otherwise indicated the standard chemicals and reagents were purchased from Roth, Sigma or Merck (Darmstadt, Germany).

5. Results

5.1. Expression patterns and the role of the CadF protein in *Campylobacter jejuni* and *Campylobacter coli*

5.1.1. Immunodetection of CadF in *Campylobacter* isolates

The binding of *Campylobacter jejuni* and *Campylobacter coli* to human fibronectin (Fn), a component of the extracellular matrix, is mediated by a 37 kDa outer-membrane protein termed CadF for *C*ampylobacter adhesion to Fn (Konkel *et al.,* 1997; Konkel *et al.,* 1999a; Konkel *et al.,* 2005). *CadF* is a single-copy, highly-conserved chromosomal encoded gene of multiple *Campylobacter* species (Konkel *et al.,* 1999a; Parkhill *et al*., 2000; Fouts *et al*., 2005; Hofreuter *et al*., 2006). Previous work based on immunoblot analysis of clinical isolates indicated that the CadF protein is highly conserved among *C. jejuni* strains from the USA (Konkel *et al.,* 1997; Konkel *et al.,* 1999a). Therefore, a variety of assays could be developed based on the detection of the *cadF* pathogenicity factor gene and its product. The first aim of the present study was to determine the genetic and functional diversity of CadF protein among *Campylobacter* strains in Germany. For this purpose the CadF proteins of a large number of *C. jejuni* and *C. coli* isolates of human and animal origin were compared and the role of CadF in the attachment and internalization of INT-407 epithelial cells was determined.

The 58 *Campylobacter* isolates, provided by cooperation partners (Table 2), were characterized as *C. jejuni* (40 strains) and *C. coli* (18 strains). Species identification, performed by cooperation partners, was based on biochemical tests (catalase, oxidase, urease activity, hippurate and indoxyl acetate hydrolysis, sensitivity to cephalothin and nalidixic acid), and a multiplex PCR assay (Cloak & Fratamico, 2002; Oyarzabal *et al.,* 2005). The *C. jejuni* isolates included strains isolated from both humans and animals, while the *C. coli* strains were all recovered from animals (Table 8).

To investigate the expression of CadF in these strains, α-CadF-1, an antibody against a conserved sequence in this protein was generated and applied in Western blot experiments along with the α-CadF-2 antibody raised against gel-purified CadF protein (Konkel *et al.,* 1997). Using these two CadF-specific antisera, a 37 kDa band (p37) and a less prominent 32 kDa band (p32) were detected in *C. jejuni* strains by immunoblotting of total cell lysates. These bands corresponded to previously described CadF protein species (Konkel *et al.,* 1997; Mamelli *et al.,* 2006; 2007). While p37 was present in all *C. jejuni* isolates, five human isolates and one from a calf failed to exhibit the less prominent p32 band (Table 8). A representative immunoblot of several *C. jejuni* isolates is shown in Fig. 3A.

Table 8. *C. jejuni* and *C. coli* isolates used in the study and detection of CadF proteins.

Species	Origin	Strain designation	Presence of CadF protein bands	
			37 kDa(p37)	32 kDa (p32)
C. jejuni	Human, feces	ATCC 43431	+	+
		NCTC 11168	+	+
		81-176	+	+
		1543/01	+	+
		ST3046	+	+
		81116	+	+
		F38011	+	+
		CDC 2004-341	+	+
		158/96	+	-
		157/96	+	-
		51/89	+	-
		230205ZH0017	+	-
		230205ZH0018	+	-
	Chicken, intestine	G 447	+	+
		G 448	+	+
		G 450	+	+
		G 451	+	+
		G 464	+	+
		G 465	+	+
		G 467	+	+
		G 477	+	+
		G 478	+	+
		G 479	+	+
		G 481	+	+
		G 482	+	+
		G 487	+	+
		G 500	+	+
		G 506	+	+
	Chicken, cloaca	RM1849	+	+
	Chicken carcass	RM1221	+	+
	Chicken, liver	151003ZH0099	+	+
	Poultry, feces	1991	+	+
	Turkey	201004ZH0078	+	+
		503	+	+
		av245	+	+
	Cat, feces	ALK 1116	+	+
	Calf, feces	ATCC 43430	+	+
	Calf, abomasum	C 130	+	-
	Cow, milk	73 Di	+	+
		100204ZH0021	+	+
C. coli *	Pig, feces	ALK 1158	+	+
		ALK 1179	+	-
		ALK 1184	+	+
		ALK 1185	+	-
		ALK 1187	+	+
		ALK 1290	+	-
		ALK 1295	+	+
		ALK 1233	+	+
		ALK 1282	+	-
	Chicken, intestine	G 427	+	+
		G 472	+	+
	Poultry, feces	Han35	+	+
		Han36	+	+
		Han135	+	+
		2371	+	+
	Poultry, liver	K1102/03	+	+
	Quail, intestine	G 510	+	-
	Turkey	av352	+	-

* In these strains the CadF protein is slightly larger (39 kDa and 34 kDa, respectively).

To verify the specificity of our α-CadF antibodies, two isogenic *cadF* mutants in *C. jejuni* strains 81116 (Krause-Gruszczynska *et al.,* 2007) and F38011 (Konkel *et al.,* 1997) were tested. These mutants lacked the p37 and p32 bands observed for the parental strains (Fig. 3C and Fig. 7A, arrows). As expected, whole-cell extracts of *C. fetus*, *H. pylori* or *E. coli* controls did not react with the CadF-specific antisera (data not shown). Equivalent amounts of proteins present were confirmed by Coomassie staining for all tested strains (Fig. 3B, D).

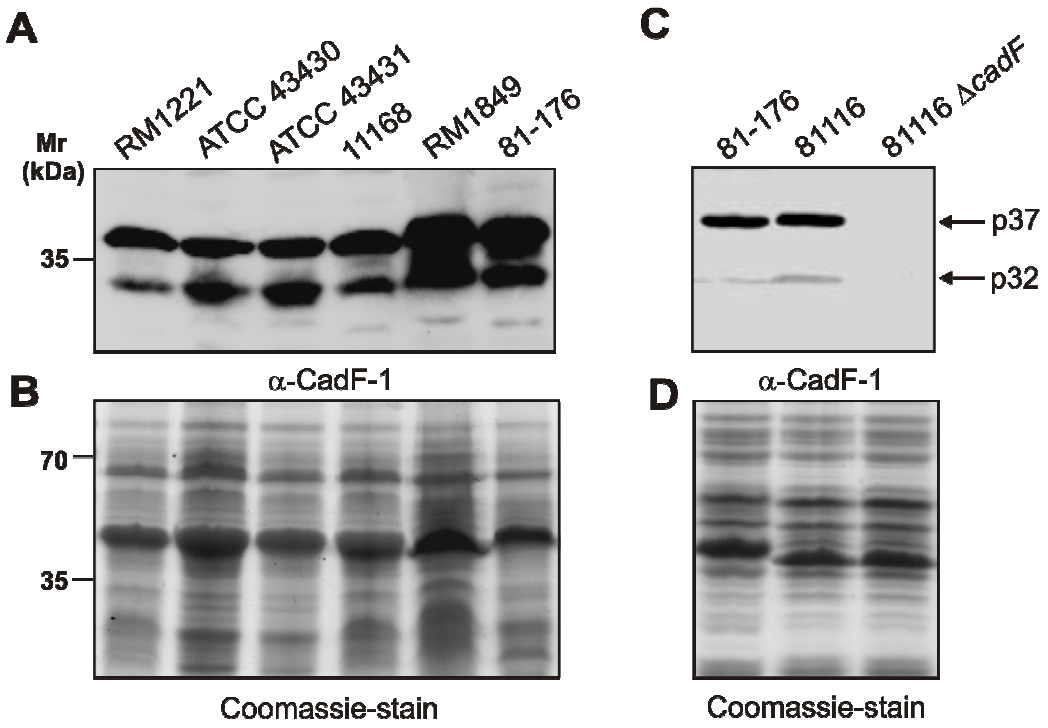


Fig. 3. Representative immunoblot analysis demonstrating CadF immunoreactivity with the α-CadF-1 antibody among *C. jejuni* isolates. (A) *C. jejuni* isolates showing 32 kDa and 37 kDa bands corresponding to the CadF proteins. (C) These bands were not detected in 81116Δ*cadF* mutant (arrows). (B, D) Coomassie stainings presenting equivalent amounts of protein in each lane.

5.1.2. Variability of CadF proteins among *C. jejuni* and *C. coli* isolates

Although the pattern of α-CadF-1 and α-CadF-2 antibodies reactivity was largely identical among the isolates, the number and intensities of the CadF protein species slightly varied among *C. jejuni* strains (Fig. 4A, arrows and asterisks), despite loading equivalent amounts of proteins (Fig. 4B). In some cases, intermediate CadF bands of ~34 kDa were also observed (Fig. 4A, arrows). Interestingly, in all *C. coli* isolates tested, CadF was slightly larger and had a weaker expression, as judged from Western blot analysis (Fig. 5A). All *C. coli* isolates exhibited a 39 kDa band (p39), while a lower migrating 34 kDa band (p34) was detected in 12 out of 18 *C. coli* strains (Fig. 5A, Table 8).

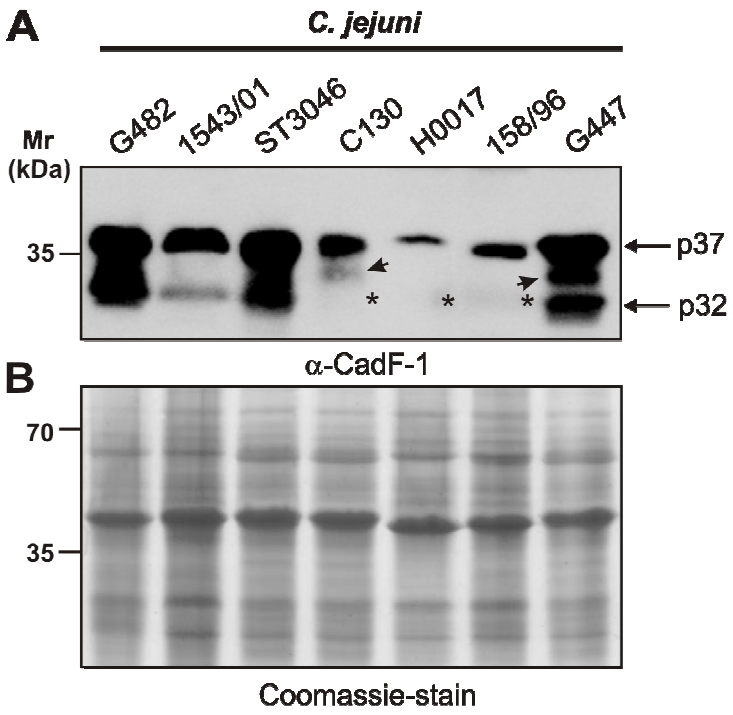
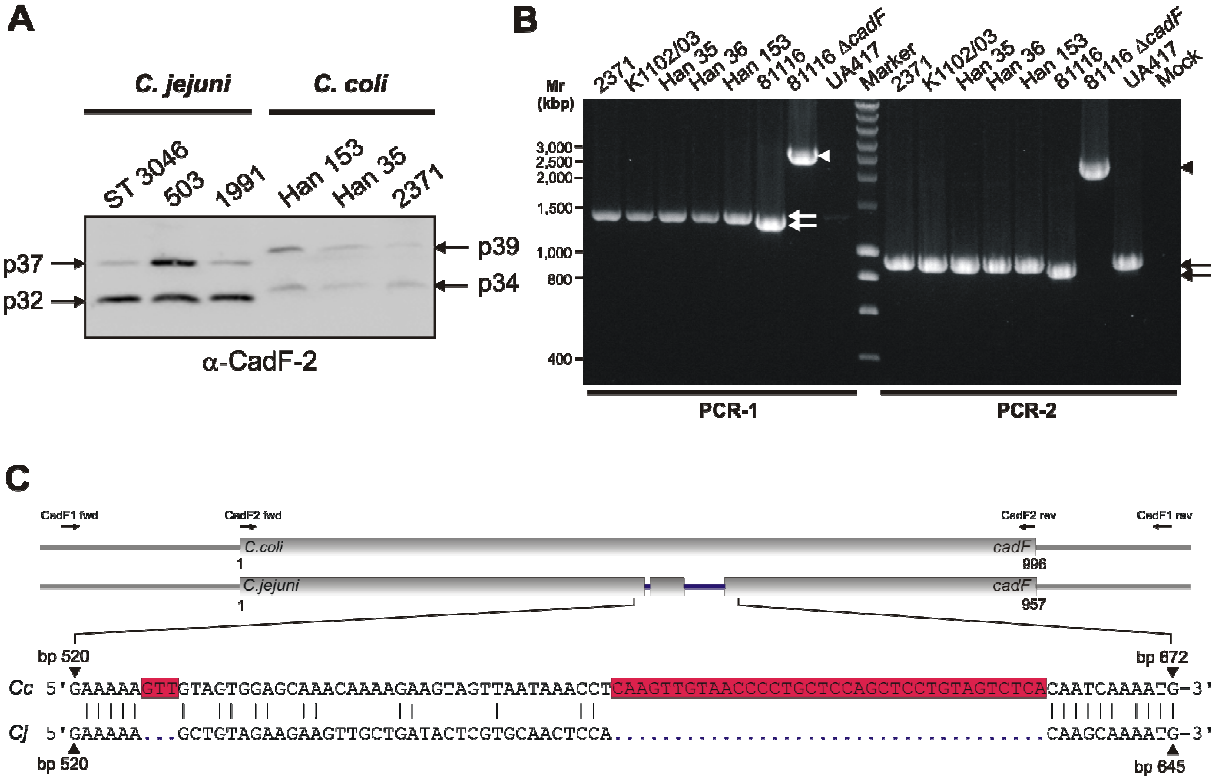


Fig. 4. Variability in number and band intensities of CadF proteins in *C. jejuni*. (A) Immunoblot analysis showing the variability in α-CadF-1 staining among strains. Arrows indicate additional bands in the pattern and asterisks indicate bands which are absent in some strains. (B) Coomassie staining presenting equivalent amounts of protein in each lane.

5.1.3. PCR amplification and sequencing of *cadF* genes

To elucidate the differences in CadF protein size and expression between *C. jejuni* and *C. coli* strains, the sequence analysis on a set of *cadF* genes was performed. PCR analysis of the *C. coli* strains, performed by cooperation partner Like van Alphen, (Utrecht University, The Netherlands) revealed a slightly larger *cadF* than that of *C. jejuni* 81116 (1320 bp versus 1285 bp for *cadF* and some flanking sequence, respectively) (Fig. 5B, arrows). PCR with primers directed against the most conserved parts within the *cadF* gene yielded 930 bps for *C. coli* strains and 890 bps for *C. jejuni* 81116 (Fig. 5B, arrows). Insertion of a kanamycin resistance cassette in 81116Δ*cadF* mutant resulted in a 1.5 kb increase in product size in both PCRs, as expected (Fig. 5B, arrowheads).

Sequencing of the *cadF* coding region from three *C. coli* isolates consistently revealed an additional sequence (39 bp) at the indicated position compared to *cadF* of *C. jejuni* (Fig. 5C). Analysis of the *cadF* sequences from three *C. jejuni* and one *C. coli* available sequenced genomes (Parkhill *et al*., 2000; Fouts *et al*., 2005; Hofreuter *et al*., 2006) confirmed these findings. Alignment of deduced amino acid sequences showed that the CadF protein from *C. coli* strains is 13 amino acids larger than those from *C. jejuni*, in agreement with the size differences seen in the Western blots (Fig. 5D).



D

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Cc 2371          MRKLLLCLGLASVLFGADNNVKFEITPTLNYNYFEGNLDMDNRYAPGIRLGYHFDDFWLDQLELGLEHYS 70
Cc HAN153        MRKLLLCLGLASVLFGADNNVKFEITPTLNYNYFEGNLDMDNRYAPGIRLGYHFDDFWLDQLELGLEHYS 70
Cc RM2228        MRKLLLCLGLASVLFGADNNVKFEITPTLNYNYFEGNLDMDNRYAPGIRLGYHFDDFWLDQLELGLEHYS 70
Cc HAN35         MRKLLLCLGLASVLFGADNNVKFEITPTLNYNYFEGNLDMDNRYAPGIRLGYHFDDFWLDQLELGLEHYS 70
Cj NCTC11168     MKKIFLCLGLASVLFGADNNVKFEITPTLNYNYFEGNLDMDNRYAPGIRLGYHFDDFWLDQLEFGLEHYS 70
Cj RM1221        MKKIFLCLGLASVLFGADNNVKFEITPTLNHNYFEGNLDMDNRYAPGIRLGYHFDDFWLDQLEFGLEHYS 70
Cj 81-176        MKKILLCLGLASVLFSADNNVKFEITPTLNYNYFEGNLDMDNRYAPGIRLGYHFDDFWLDQLEFGLEHYS 70
                 *:*::**********.**************:**********************************:******

Cc 2371          DVKYTNSTLTIDITRTYLSAIKGIDLGEKFYFYGLACVGYEDFSKCAFDNKSCGFCHYGAGLKFRLSDSL 140
Cc HAN153        DVKYTNSTLTIDITRTYLSAIKGIDLGEKFYFYGLAGGGYEDFSKGAFDNKSGGFGHYGAGLKFRLSDSL 140
Cc RM2228        DVKYTNSTLTIDITRTYLSAIKGIDLGEKFYFYGLAGVGYEDFSKGAFDNKSGGFGHYGAGLKFRLSDSL 140
Cc HAN35         DVKYTNSTLTIDITRTYLSAIKGIDLGEKFYFYGLAGGGYEDFSKGAFDNKSGGFGHYGAGLKFRLSDSL 140
Cj NCTC11168     DVKYTNTNKTIDITRTYLSAIKGIDVGEKFYFYGLACGGYEDFSNAAYDNKSCGFCHYGAGVKFRLSDSL 140
Cj RM1221        DVKYTNTNKTIDITRTYLSAIKGIDVGEKFYFYGLAGGGYEDFSNAAYDNKSGGFGHYGAGVKFRLSDSL 140
Cj 81-176        DVKYTNTNKTIDITRTYLSAIKGIDVGEKFYFYGLAGGGYEDFSNAAYDNKSGGFGHYGAGVKFRLSDSL 140
                 ******:. ***************:********** ******:.*:*************:********

Cc 2371          ALRLETRDQISFHDADHSWVSTLGISFGFGAKQEKVVVEQTKEVVNKPQVVTPAPAPVVSQSKCPEEPRE 210
Cc HAN153        ALRLETRDQISFHDADHSWVSTLGISFGFGAKQEKVVVEQTKEVVNKPQVVTPAPAPVVSQSKCPEEPRE 210
Cc RM2228        ALRLETRDQISFHDADHSWVSTLGISFGFGAKQEKVVVEQTKEVVNKPQVVTPAPAPVVSQSKCPEEPRE 210
Cc HAN35         ALRLETRDQISFHDADHSWVSTLGISFGFGAKQEKVVVEQTKEVVNKPQVVTPAPAPVVSQSKCPEEPRE 210
Cj NCTC11168     ALRLETRDQINFNHANHNWVSTLGISFGFGGKKEK-AVEEVADTRATP------------QAKCPVEPRE 197
Cj RM1221        ALRLETRDQINFNHANHNWVSTLGISFGFGGKKEK-AVEEVADTRATP------------QVKCPVEPRE 197
Cj 81-176        ALRLETRDQINFNHANHNWVSTLGISFGFGGKKEK-AVEEVADTRPAP------------QAKCPVEPRE 197
                 **********.*:.*:*.************.*:** .**:. :.   *            * *** ****

Cc 2371          CALLDENGCEKTIYLEGHFDFDKVNINPAFEEQIKEIAQILDENVRHDTILECHTDNIGSRSYNQKLSER 280
Cc HAN153        GALLDENGCEKTIYLEGHFDFDKVNINPAFEEQIKEIAQILDENVRYDTILEGHTDNIGSRSYNQKLSER 280
Cc RM2228        GALLDENGCEKTIYLEGHFDFDKVNINPAFEEQIKEIAQILDENVRYDTILEGHTDNIGSRSYNQKLSER 280
Cc HAN35         GALLDENGCEKTIYLEGHFDFDKVNINPAFEEQIKEIAQILDENVRYDTILEGHTDNIGSRSYNQKLSER 280
Cj NCTC11168     CALLDENGCEKTISLEGHFGFDKTTINPTFQEKIKEIAKVLDENERYDTILECHTDNIGSRAYNQKLSER 267
Cj RM1221        GALLDENGCEKTISLEGHFGFDKTTINPTFQEKIKEIAKVLDENERYDTILEGHTDNIGSRAYNQKLSER 267
Cj 81-176        GALLDENGCEKTISLEGHFGFDKTTINPTFQEKIKEIAKVLDENERYDTILEGHTDNIGSRAYNQKLSER 267
                 ************ *****.***..***:*:*:*:*****:;**** *:**************:********

Cc 2371          RANSVAKELEKFGVDKSRIQTVGYGQDKPRSSNDTKEGRADNRRVEAKFILN 332
Cc HAN153        RANSVAKELEKFGVDKSRIQTVGYGQDKPRSSNETKEGRADNRRVDAKFILR 332
Cc RM2228        RANSVAKELEKFGVDKSRIQTVGYGQDKPRSSNDTKEGRADNRRVDAKFILR 332
Cc HAN35         RANSVAKELEKFGVDKSRIQTVGYGQDKPRSSNDTKEGRADNRRVESLVNSR 332
Cj NCTC11168     RAKSVANELEKYGVEKSRIKTVGYGQDNPRSSNDTKEGRADNRRVDAKFILR 319
Cj RM1221        RAKSVANELEKYGVEKSRIKTVGYGQDNPRSSNDTKEGRADNRRVDAKFILR 319
Cj 81-176        RAKSVANELEKYCVEKSRIKTVGYGQDNPRSSNDTKECRADNRRVDAKFILR 319
                 **:***:****:**:****:*******:*****:**********:: . .
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Fig. 5. Refers to the figure on page 50. Different CadF protein sizes and expression levels in *C. jejuni* and *C. coli* strains. (A) Representative immunoblot analysis with α-CadF-2 antibody showing that CadF proteins of *C. coli* strains are slightly larger and less immuno-reactive than *C. jejuni* CadF (arrows). (B) PCR analysis of *cadF* genes in *C. jejuni* and *C. coli* isolates by agarose gel elecrophoresis using two sets of primers (PCR-1 and PCR-2). Arrows indicate the difference in size of *C. coli* amplified products as compared to the *C. jejuni* 81116 control. Insertion of a kanamycin resistance cassette in 81116Δ*cadF* mutant resulted in expected increase in product size (arrowheads). (C) Sequencing of the PCR products revealed insertion sequences in the *C. coli cadF* genes at the indicated positions. An extra 39 bp sequence was detected in the *C. coli cadF* genes (indicated in red). (D) Alignment of the deduced amino acid sequences coding for CadF proteins showing differences between *C. jejuni* and *C. coli* strains. Extra 13 amino acids in the *C. coli* sequences are indicated in red. Symbols: *, identical amino acids; :, conserved substitution; ., semi-conserved substitution.

5.1.4. Binding and invasion of INT-407 cells by differently CadF-expressing *C. jejuni* and *C. coli* strains

Possible differences in bacterial adhesion and invasion between the CadF-expressing *C. jejuni* and *C. coli* isolates were explored in infection assays with INT-407 cells. These non-phagocytic intestinal epithelial cells are widely used as an *in vitro* model system to study *Campylobacter* invasion (Hu & Kopecko, 1999; Biswas *et al.,* 2000; Konkel *et al*., 2004; Hu *et al*., 2005). Quantification of cell-associated (Fig. 6A) and intracellular bacteria (Fig. 6B) by the gentamicin protection assay revealed that the *C. jejuni* isolates expressing p37 CadF exhibited significantly higher binding and invasion rates than *C. coli* strains expressing p39 CadF ($P \leq 0.001$). The *C. coli* isolates Han35 and Han153 exhibited the lowest values of cell-associated and intracellular bacteria.

To determine the overall contribution of the CadF protein in the binding and invasion of *C. jejuni* to INT-407 cells, the interactions of *C. jejuni* 81116Δ*cadF* and F38011Δ*cadF* mutants and their respective wild-type strains with cells were examined. Immunoblot analysis confirmed that the CadF protein was not synthesized by either *cadF* mutant strain (Fig. 7A). Quantification of cell-associated (Fig. 7B) and intracellular bacteria (Fig. 7C) by the gentamicin protection assay revealed that the *cadF* mutants exhibited lower binding and invasion rates than wild-type *C. jejuni*. The 81116Δ*cadF* mutant showed 49% reduction in adherence and 36.5% in invasion when compared to its wild-type strain 81116, whereas F38011Δ*cadF* showed 64% reduction in adherence and 60% in invasion when compared to the F38011 wild-type (Fig. 7B, C). These data were statistically significant ($P \leq 0.001$). These findings demonstrate that CadF is an important pathogenicity factor of *C. jejuni*. The data also show that CadF is not only important for bacterial binding to host cells, as suggested recently (Konkel *et al.,* 2005) but also for host cell entry.

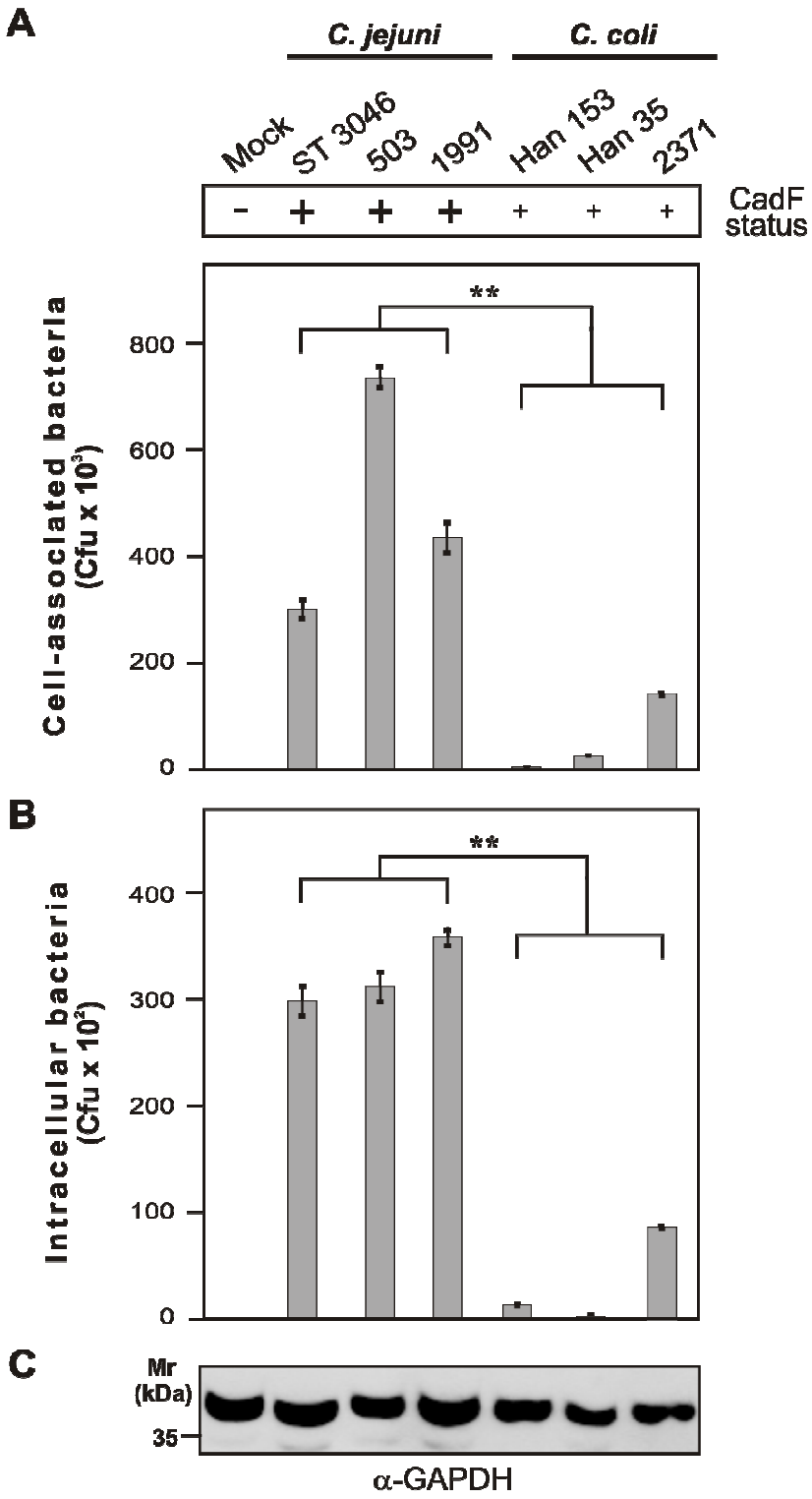


Fig. 6. Effect of CadF on adhesion to and invasion of *C. jejuni* and *C. coli* isolates. INT-407 cells were infected for 6 hrs with *C. jejuni* and *C. coli* strains. (A) Total cell associated and (B) intracellular *Campylobacter* cells were quantified by gentamicin protection assays. (**) Statistically significant ($P \leq 0.001$). (C) To ensure equal numbers of INT-407 cells, each infection was performed in duplicate including one sample for the GAPDH house keeping protein control immunostaining.

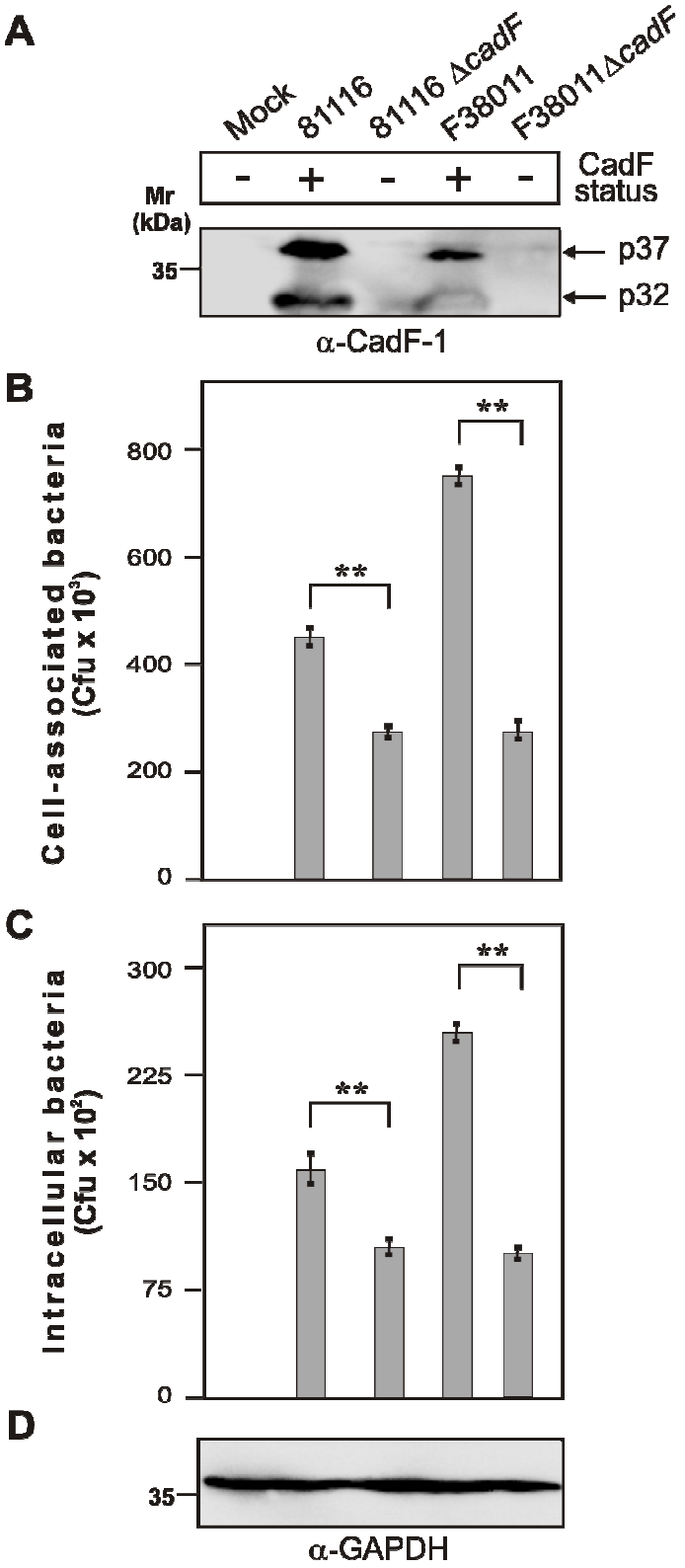


Fig. 7. Effect of CadF expression on adhesion and invasion of *C. jejuni*. INT-407 cells were infected for 6 hrs with wild-type 81116 *vs.* 81116Δ*cadF* and wild-type F38011 *vs.* F38011Δ*cadF*. (A) The expression of CadF proteins during infection was verified by immunoblotting using the α-CadF-1 antibody. (B) Total cell associated and (C) intracellular *Campylobacter* cells were quantified by gentamicin protection assays. (**) Statistically significant ($P \leq 0.001$). (D) To ensure equal numbers of INT-407 cells, each infection was performed in duplicate including one sample for the GAPDH house keeping protein control immunostaining.

5.2. Role of the small Rho GTPases Rac1 and Cdc42 in host cell invasion of *Campylobacter jejuni*

5.2.1. *Campylobacter jejuni* invasion is time-dependent and associated with dynamic host actin cytoskeletal rearrangements

Since *C. coli* exhibited only weak invasiveness as compared to *C. jejuni* and was independent of CadF, further studies concentrated on *C. jejuni*. Most of these studies were performed with *C. jejuni* 81-176, a model strain used world-wide as described in chapter 3.4. To investigate the *C. jejuni* entry process in more detail, the interaction of 81-176 with the surface of INT-407 epithelial cells was analyzed by high resolution field emission scanning electron microscopy (FESEM), performed by cooperation partner Dr. M. Rohde (Helmholtz Center for Infection Research, Braunschweig, Germany). FESEM analysis revealed that the bacteria were able to attach to the host cell surface, followed by cellular invasion of the *C. jejuni* which was observed predominantly after 4-6 hrs of infection (Fig. 8A, red arrows). Interestingly, it was found that the bacterium invaded into the cell in a very specific manner first with its tip followed by the flagellar end (Fig. 8A-B, yellow arrowheads). Tight engulfment and membrane ruffles were also observed regularly, suggesting the occurrence of GTPase activation followed by dynamic membrane rearrangements during the invasion process (Fig. 8B, blue arrows).

To determine the time frame required for *C. jejuni* to enter the eukaryotic cells, INT-407 monolayers were infected for different time periods, ranging from 30 min to 24 hrs, and the number of intracellular bacteria was then determined by gentamicin protection assays (Fig. 9A). The results indicated that penetration of *C. jejuni* into cultured cells occurred as early as 30 min after infection and that the number of intracellular bacteria increased rapidly between 4 to 6 hrs. These data are in good agreement with those obtained by FESEM. Intracellular persistence of *C. jejuni* could be shown by the fact that the number of intracellular bacteria even increased during 24 hrs infections. However, survival of the internalized *C. jejuni* past 24 hrs could not be assessed because the infected INT-407 cells started detaching after this time period.

The data obtained with the gentamicin protection assays and FESEM were then confirmed by confocal microscopy examination. For example, INT-407 cells infected with GFP-expressing *C. jejuni* also revealed numerous intracellular bacteria (Fig. 9B-E, arrows). Interestingly, immunofluorescence staining with rhodamine-conjugated phalloidin indicated condensation of actin filaments around and beneath the adherent or entering bacteria (Fig. 9B-E, arrows). This result is consistent with previous observations (Konkel *et al.,* 1992; Monteville *et al.,* 2003). In addition, a significant proportion of intracellular *C. jejuni* was also

observed to be closely associated with condensed patches of host filamentous actin (Fig. 9F-H, arrowheads). These findings support the view, in addition to previous reported microtubule-dependent invasion (Oelschlaeger *et al.,* 1993), that *C. jejuni* induces actin-cytoskeletal rearrangements and Rho GTPase activation.

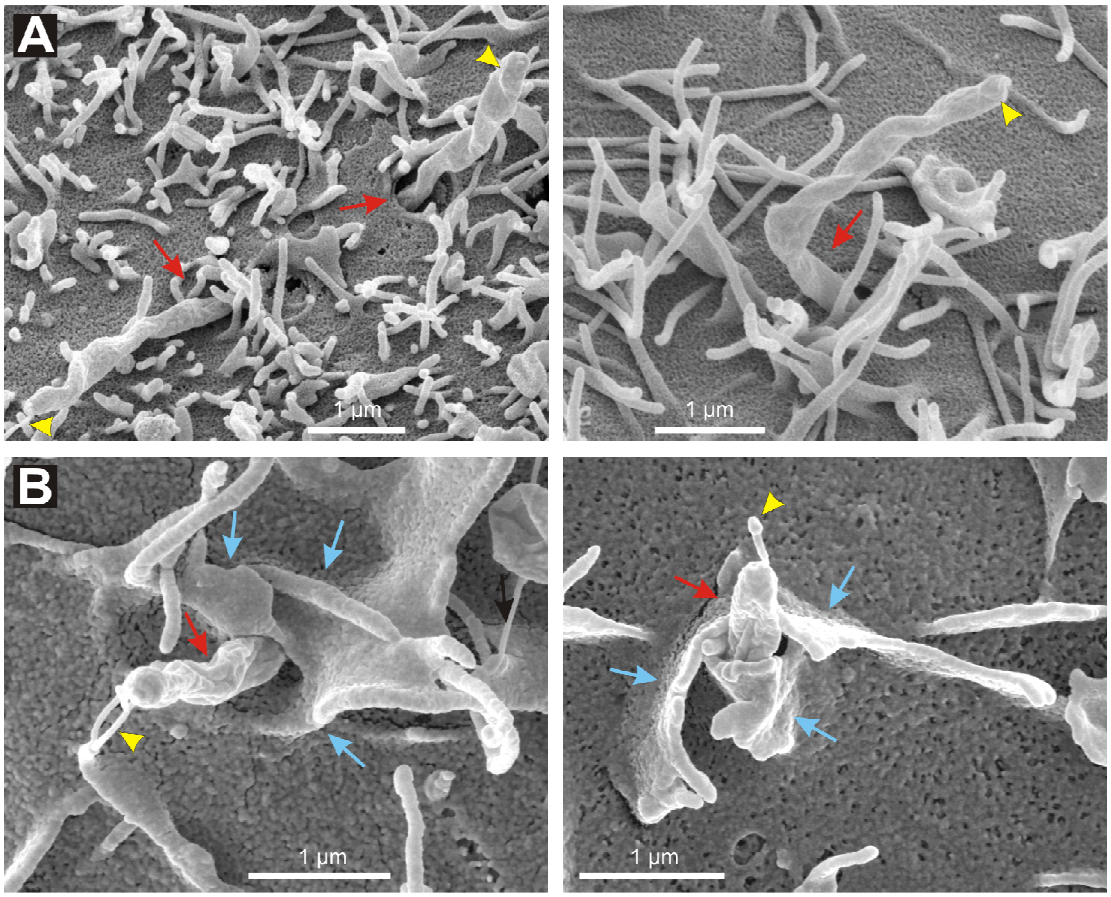


Fig. 8. High resolution field emission scanning electron microscopy of *C. jejuni* invasion into INT-407 epithelial cells. (A) *C. jejuni* 81-176 (red arrows) infected for 4-6 hrs were able to induce their entry into the eukaryotic target cells and (B) were often associated with membrane ruffles (blue arrows) and tight engulfment of the bacteria. The bacteria invaded into the cell first with their tip followed by the other end carrying the flagella (yellow arrowheads).

5.2.2. Inactivation of Rac and Cdc42, but not RhoA, prevents *C. jejuni* internalization

Internalization of bacterial pathogens in general has typically been observed to induce rearrangement of the host actin-cytoskeletal structure caused by activation of small Rho family GTPases (Hardt *et al.,* 1998; Criss *et al.,* 2001; Kazmierczak *et al.,* 2001; Cossart & Sansonetti, 2004; Rottner *et al.,* 2004; Pizarro-Cerda & Cossart, 2006), but their importance and role in infections with *C. jejuni* has not been investigated yet.

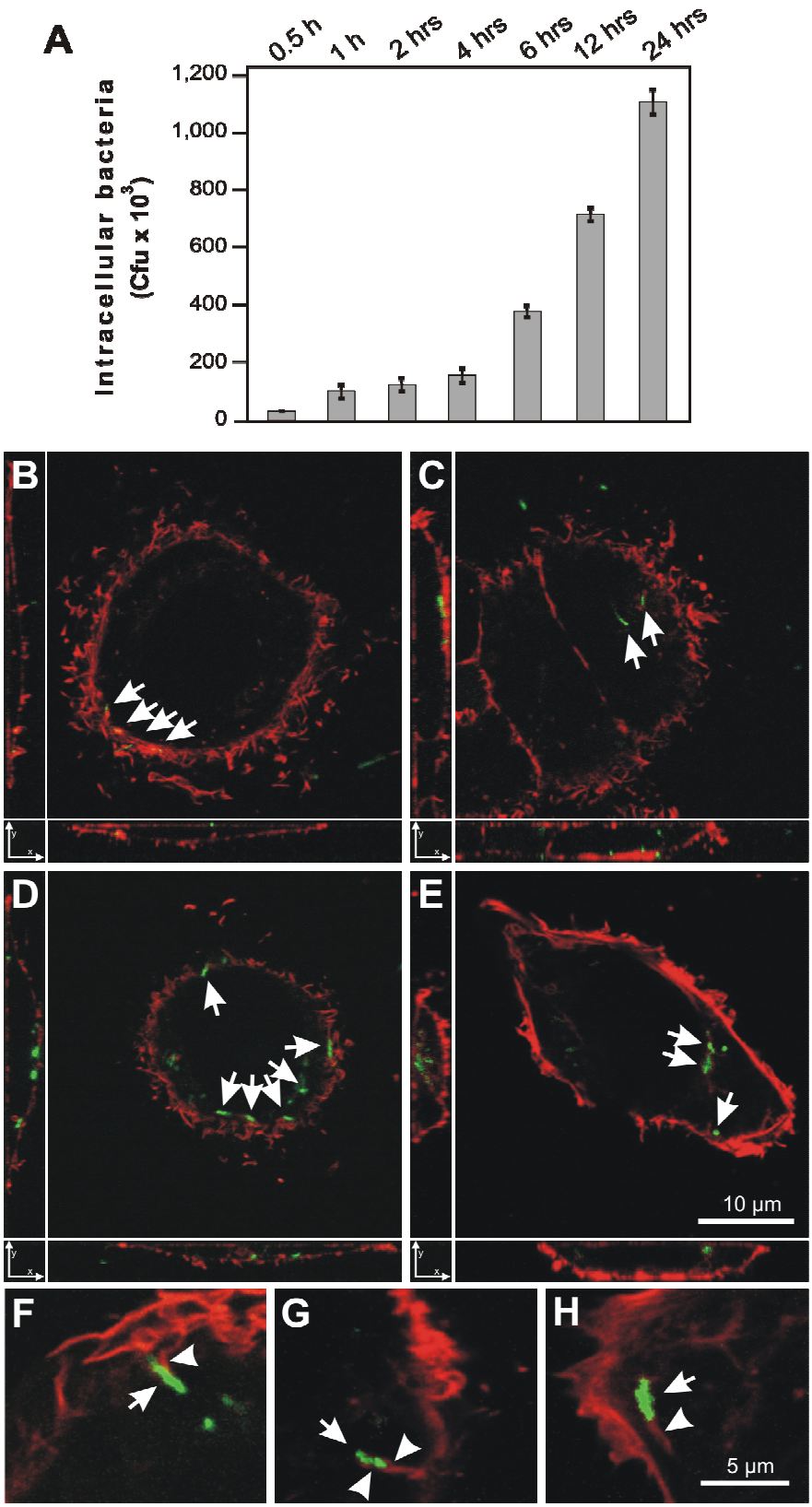


Fig. 9. Quantification and confocal scanning microscopy of *C. jejuni* invasion. (A) INT-407 cells were infected with *C. jejuni* for the indicated periods of time and the numbers of intracellular bacteria were quantified by gentamicin protection assay. To directly visualize the internalized bacteria the INT-407 cells were infected with GFP-expressing *C. jejuni* for (B, C) 4 hrs or (D, F-H) 24 hrs and stained the cells with rhodamine-phalloidin (red). Invading or internalized bacteria are labeled with arrows. (F-H) Enlarged micrographs show the proximity between internalized bacteria and filamentous actin (arrowheads) in the infected host cells.

To investigate if small Rho GTPases play a role in the uptake of *C. jejuni*, INT-407 cells were first incubated with the pharmacological inhibitor compactin which inactivates GTPases by blocking their isoprenylation and membrane targeting (Chong *et al.,* 1994). Indeed, exposure of INT-407 cells to compactin significantly reduced *C. jejuni* internalization as quantified by gentamicin protection assays (Fig. 10A). Next, the cells were treated with toxin B from *Clostridium difficile* prior to bacterial infection. Toxin B is a protein which mono-glucosylates Rho, Rac and Cdc42, leading to their irreversible inactivation in a very efficient manner (Barbieri & Aktories, 2005). As expected, in the presence of toxin B, INT-407 cells started rounding up and actin structures were disrupted (data not shown), leading to profound inhibition of *C. jejuni* internalization in a dose-dependent manner, as determined by gentamicin protection assays (Figs. 10A, C) and confocal microscopy (Fig. 11D). Toxin B had no effect on bacterial viability, as indicated by the cell-associated *C. jejuni* CFU (Fig. 10C). Similar results were obtained with TcdB toxin which expresses similar inactivating activities on Rho, Rac and Cdc42 (Fig. 10B). To further elucidate which GTPases are involved in *C. jejuni* invasion, another variant of toxin B from *C. difficile* strain 1470 serotype F (TcdBF) was applied which inactivates Rac and R-Ras but not Rho and Cdc42 (Chaves-Olarte *et al.,* 2003). Interestingly, TcdBF also had a pronounced blocking effect (Fig. 10B). These results suggest that Rac but not Rho is involved in the entry process of *C. jejuni*. To confirm this idea, exoenzyme C3 from *Clostridium botulinum* which specifically inactivates RhoA-C was used (Genth *et al.,* 2003). Treatment of INT-407 cells with the cell-permeable C3 fusion protein for 6 hrs resulted in the complete loss of stress fibers consistent with inhibition of Rho (Genth *et al.,* 2003 and data not shown). Infection of INT-407 cells pretreated with C3 led to a slight increase of bacterial binding but did not block *C. jejuni* internalization, excluding a role of RhoA-C in this effect (Fig. 10A). The latter result further excludes the possibility that the reduced *C. jejuni* invasion was due to mere changes of actin dynamics.

To confirm that Rac1 and Cdc42 activity was required for bacterial internalization, INT-407 cells were transiently transfected with the dominant-negative (DN) alleles of Rac1 (Rac1-T17N) or Cdc42 (Cdc42-T17N), and the empty vector as a control. Transfection of INT-407 cells with both DN-Rac1 and DN-Cdc42 resulted in a significantly reduced *C. jejuni* invasion as determined by gentamicin protection assays; expression of either DN-Rac1 or DN-Cdc42 alone was less effective (Fig. 12A). In conclusion, Rac1 and Cdc42 were required for the internalization of *C. jejuni*.

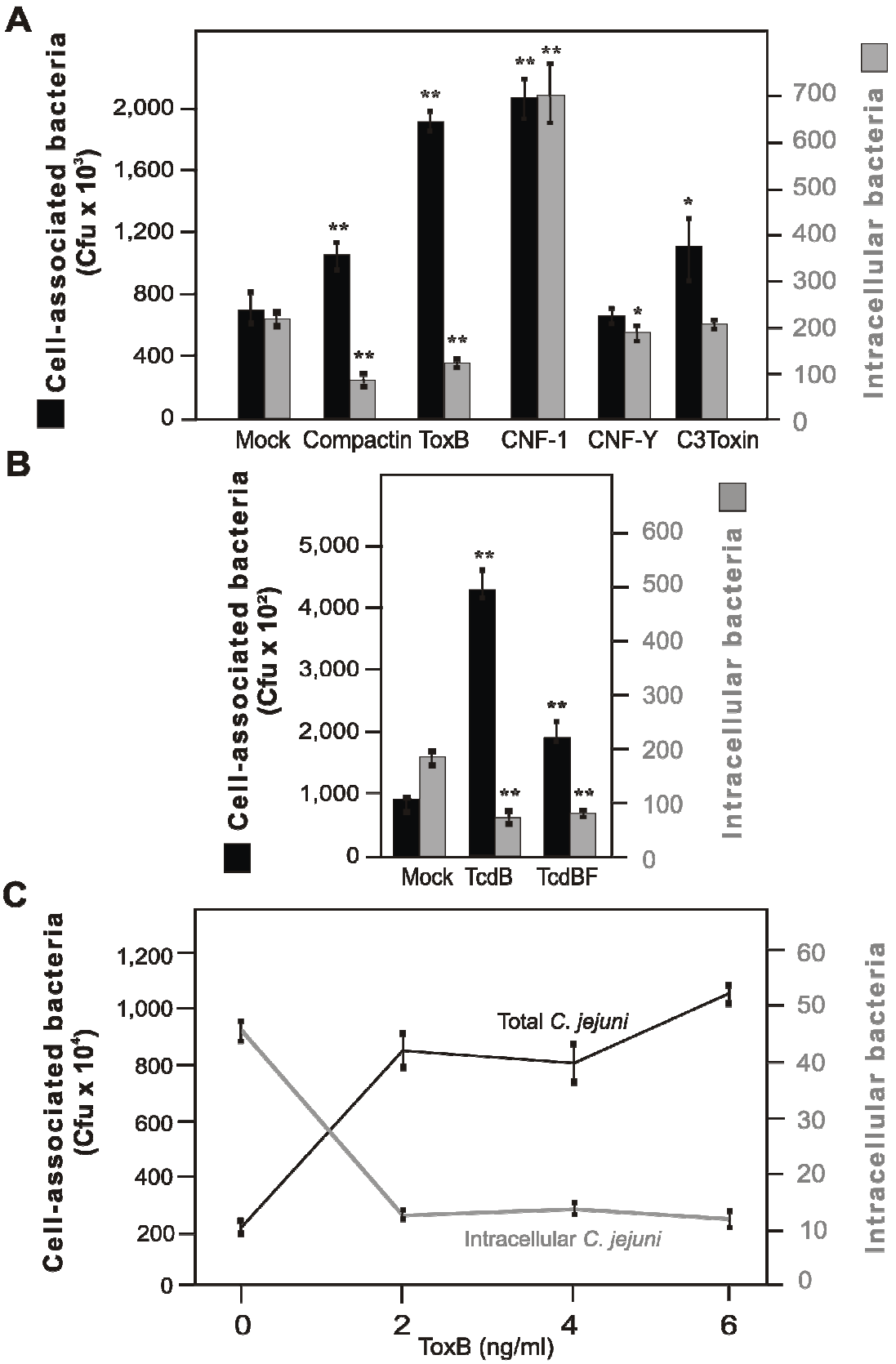


Fig. 10. Effects of pharmacological inhibitors and bacterial protein toxins targeting small Rho GTPases on host cell internalization of *C. jejuni*. (A, B) INT-407 monolayers were pre-incubated with the indicated inhibitors or toxins (Materials & Methods) and infected with *C. jejuni* for 6 hrs. Total cell associated and intracellular *C. jejuni* were quantified by gentamicin protection assays. (C) Dose-dependent inhibition of *C. jejuni* uptake by toxin B. (*) $P<0.05$ and (**) $P\leq0.005$ were considered as statistically significant as compared to the mock control.

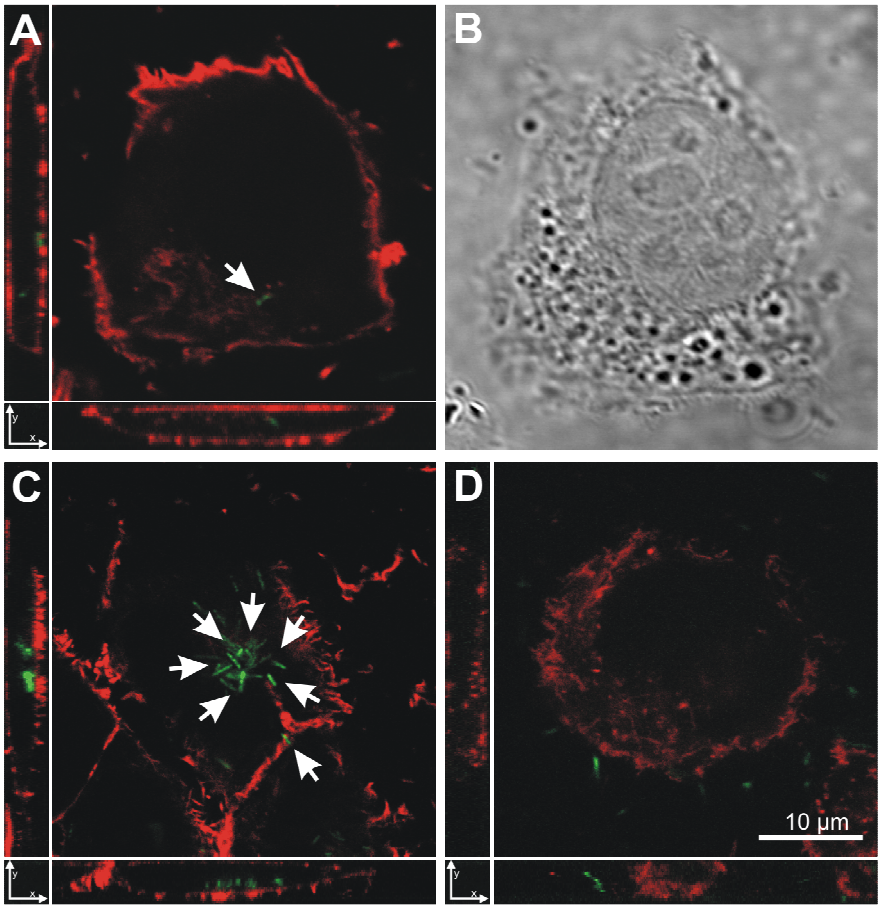


Fig. 11. Confocal scanning microscopy of *C. jejuni* invasion in the presence of GTPase-modulating toxins. (Panel A with corresponding phase contrast picture in B) INT-407 monolayers were treated with control buffer, (C) Rho GTPase activating toxin CNF-1 or (D) Rho GTPase inactivating toxin B, (Materials & Methods). Subsequently, the cells were infected with GFP-expressing *C. jejuni* for 6 hrs. Arrows indicate examples of internalized bacteria.

To prove by more direct approach whether small Rho GTPases are involved in *C. jejuni* invasion, expression of Rac1, Cdc42 or RhoA was down-regulated using target-specific small-interfering RNA (siRNA). While silencing of Rac1 and Cdc42 expression led to the significant reduction in *C. jejuni* internalization (Fig. 13A, B), both down-regulation of RhoA (Fig. 13C), and transfection with non-targeting scrambled sequence, used as mock control had no effect on *C. jejuni* uptake, as quantified by gentamicin protection assays (Fig. 13A-C). This data further confirmed that Rac1 and Cdc42 but not RhoA are involved in *C. jejuni* host cell invasion.

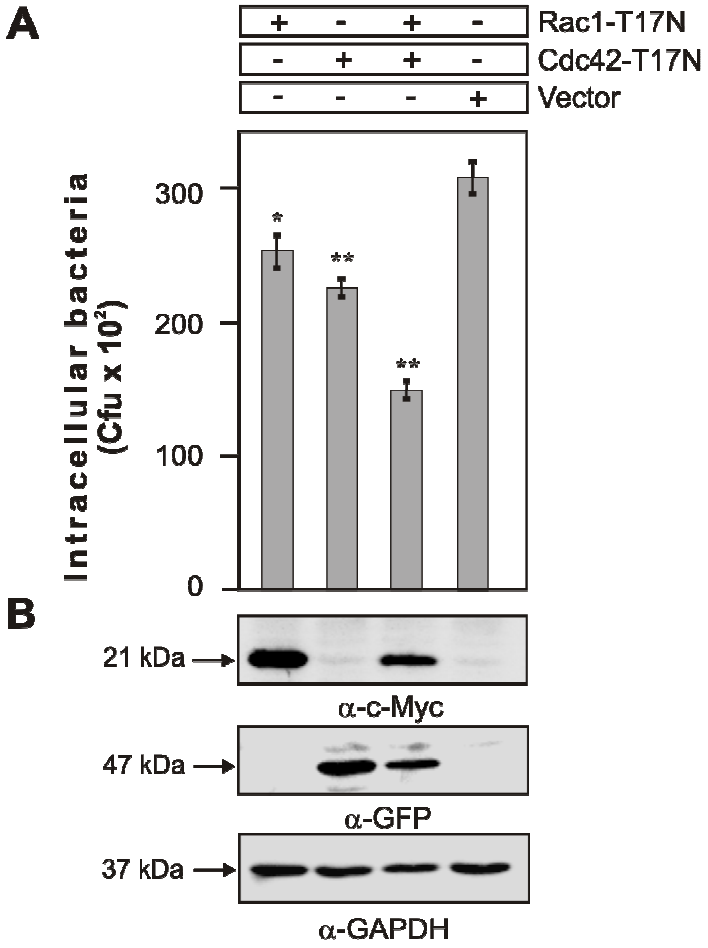


Fig. 12. Effect of expression of dominant-negative forms of Rho GTPases on *C. jejuni* uptake. Myc-tagged or GFP-tagged GTPase constructs and the empty vector control were transfected into INT-407 cells. After 48 hrs, GTPase-expressing cells were infected with *C. jejuni* for 6 hrs. (A, C) Intracellular bacteria were quantified by gentamicin protection assays. (*) $P \leq 0.05$ and (**) $P \leq 0.005$ were considered as statistically significant as compared to the mock control. (B) Expression of the individual GTPase constructs was verified by Western blot analysis using α-c-Myc and α-GFP antibodies. GAPDH expression levels were determined as control.

5.2.3. Activation of Rac1 and Cdc42, but not RhoA, promotes *C. jejuni* internalization

To investigate whether activation of Rac1 and Cdc42 trigger bacterial invasion, INT-407 cells were pre-treated with the cytotoxic necrotizing factor 1 (CNF-1), a bacterial toxin that activates Rho, Rac, and Cdc42 followed by the accumulation of stress fibers, focal adhesions, microspikes and membrane ruffles in treated cells (Flatau *et al.,* 1997; Schmidt *et al.,* 1997; Lerm *et al.,* 1999). Exposure of INT-407 cells to CNF-1 resulted in an approximately 3.3-fold increase in *C. jejuni* internalization (as compared with untreated cells), while a functionally inactive CNF-1 carrying a single point mutation (C866S) (Schmidt *et al.,* 1998) did not, thereby excluding effects other than GTPase activation (Fig. 10A and data not shown). Enhanced bacterial uptake triggered by CNF-1 was also confirmed by confocal microscopy (Fig. 11C).

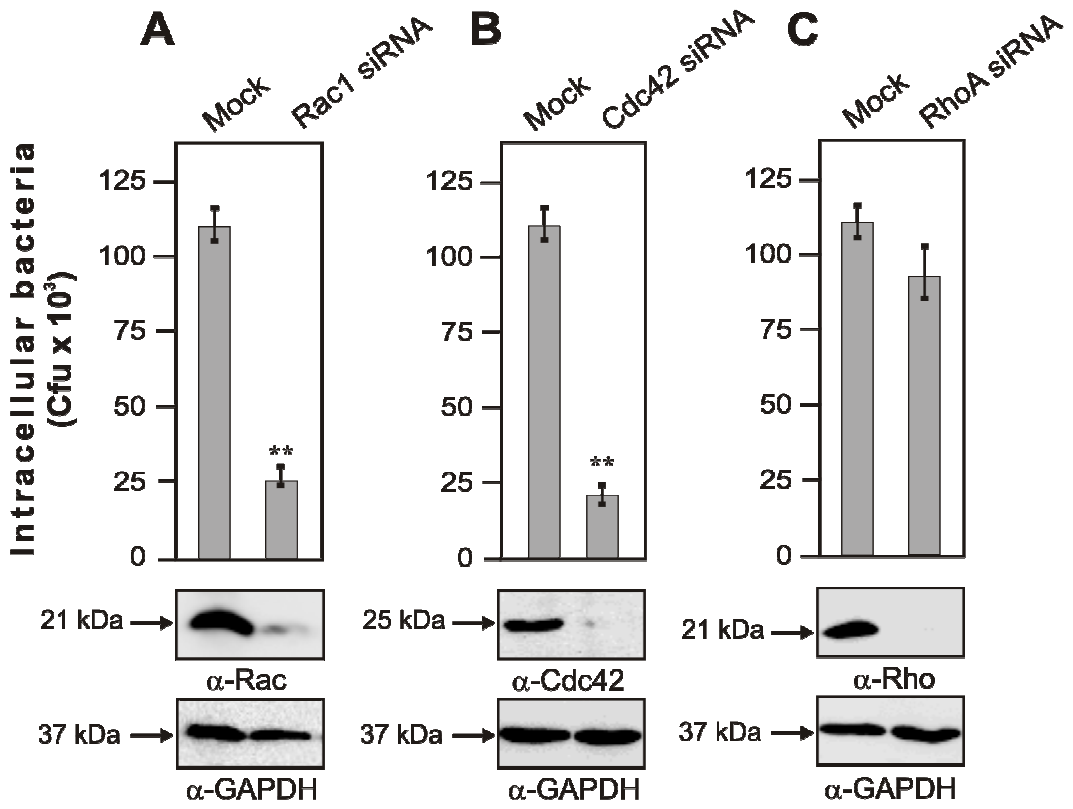


Fig. 13. Effect of knockdown of Rac1, Cdc42 or RhoA on *C. jejuni* invasion. INT-407 cells were transfected with (A) Rac1- (B) Cdc42- (C) RhoA-small interfering RNA (siRNA). After 48 hrs, cells were infected with *C. jejuni* for 6 hrs. Intracellular bacteria were quantified by gentamicin protection assays. (**) $P \leq 0.005$ were considered as statistically significant as compared to the mock control. Immunoblotting with α-Rac1, α-Cdc42, α-RhoA antibodies confirmed down-regulation of the proteins. GAPDH expression levels were determined as control.

It is noteworthy that the number of bacteria bound to epithelial cells was also significantly increased in both the CNF-1- and toxin B-treated samples as compared to the control (Fig. 10A). However, this is not surprising as the toxins markedly altered the morphology of the INT-407 cells in a manner which should improve bacterial access to the basolateral pole, where *C. jejuni* binding (and internalization) are thought to occur preferentially (Monteville & Konkel, 2002). In contrast, increased internalization of *C. jejuni* was not observed after treatment of the cells with the CNF-Y (Fig. 10A), a specific activator of RhoA (Hoffmann *et al.,* 2004; Hoffmann & Schmidt, 2004). This result strongly suggests that the elevated internalization of *C. jejuni* in CNF1-treated cells was based on activation of Rac and Cdc42.

To confirm the latter idea, INT-407 cells were transiently transfected with constitutively-active (CA) mutant alleles of Rac1 (Rac1-Q61L), Cdc42 (Cdc42-Q61L), and RhoA (RhoA-G14V), and the empty vector as a control. After 48 hrs, the transfected cells were infected with *C. jejuni* for another 6 hrs and intracellular bacteria were quantified by gentamicin protection assays (Fig. 14A). The most pronounced effect was observed with

CA-Rac1 whose expression stimulated *C. jejuni* internalization about 4.7-fold as compared to the empty vector control (P=0.003, Student's *t*-test). A moderate effect was seen with CA-Cdc42 (about 1.7-fold increase) which was also statistically significant (P=0.011). In agreement with the data obtained with CNF-Y toxin, expression of CA-RhoA resulted in a significant decrease of *C. jejuni* internalization (P=0.003, Fig. 14A). Similar expression of each of the CA-GTPase proteins in the assays was confirmed by immunoblotting using an α-c-Myc antibody with GAPDH as a control (Fig. 14B). Thus, the data suggest that activation of Rac1 and Cdc42 but not RhoA is most likely to govern *C. jejuni* invasion.

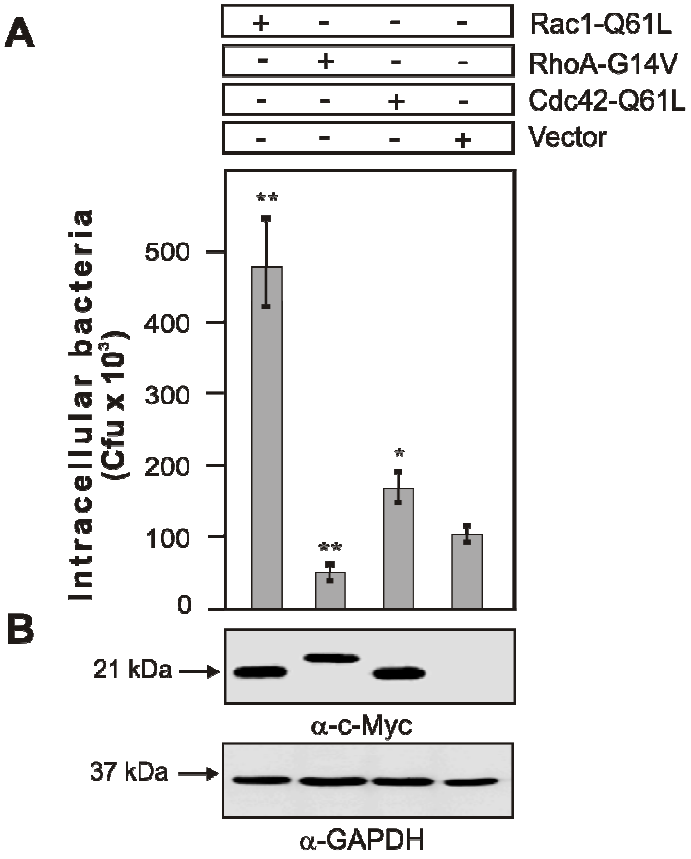


Fig. 14. Effect of expression of constitutively-active forms of Rho GTPases on *C. jejuni* uptake. Myc-tagged GTPase constructs and the empty vector control were transfected into INT-407 cells. After 48 hrs, GTPase-expressing cells were infected with *C. jejuni* for 6 hrs. (A) Intracellular bacteria were quantified by gentamicin protection assays. (*) $P \leq 0.05$ and (**) $P \leq 0.005$ were considered as statistically significant as compared to the mock control. (B) Expression of the individual GTPase constructs was verified by Western blot analysis using α-c-Myc and α-GFP antibodies. GAPDH expression levels were determined as control.

To reveal by another more direct approach whether the activity of Rac1 and Cdc42 is crucial for the cellular entry of *C. jejuni*, the presence of internalized bacteria was analyzed by confocal microscopy, in 50 individual INT-407 cells, each overexpressing the DN- or CA-GTPase proteins, respectively (Fig. 15). Internalized GFP-expressing *C. jejuni* were detected within multiple non-transfected cells of the field (data not shown).

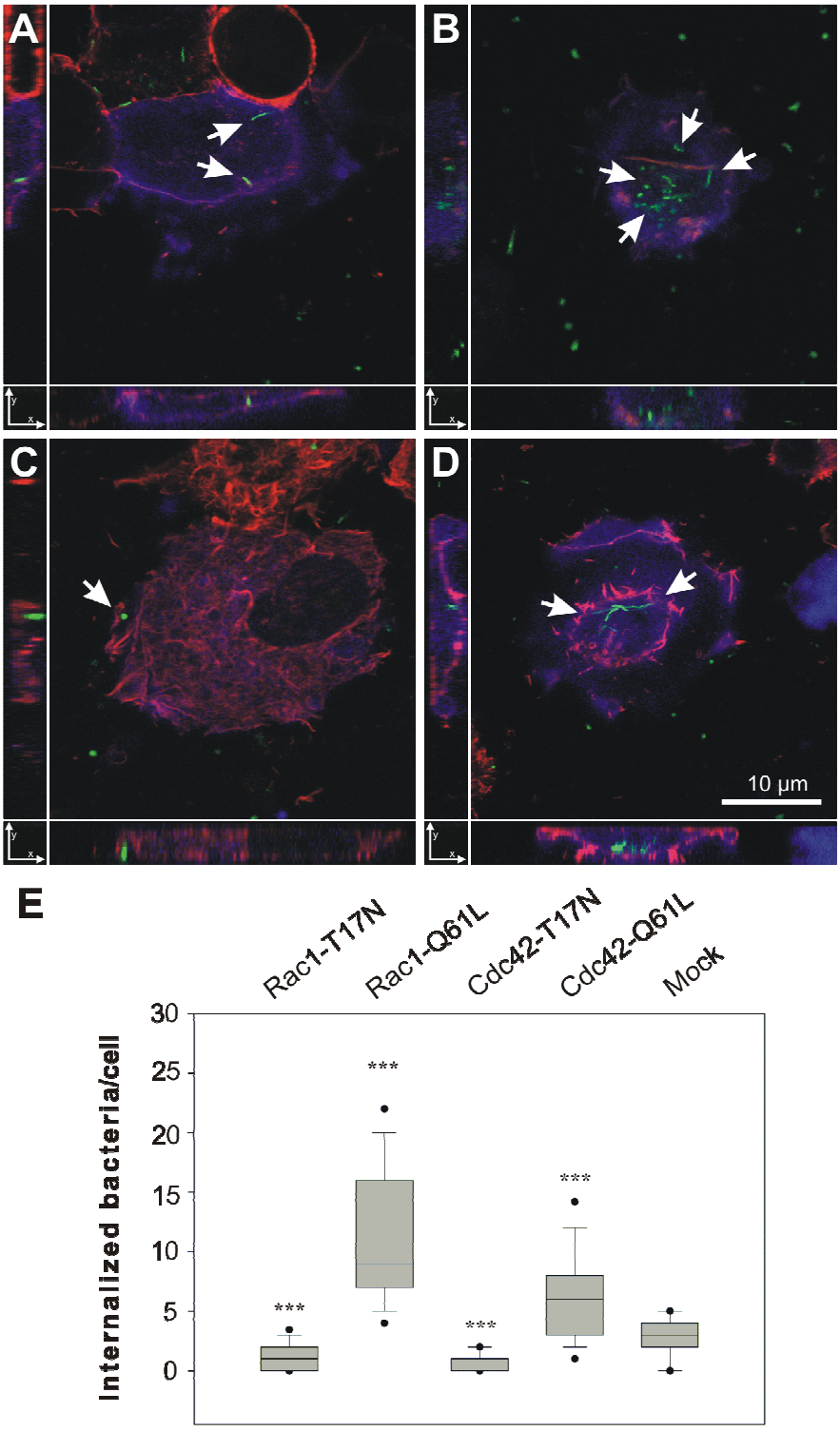


Fig. 15. Rac1 and Cdc42 activities are important for *C. jejuni* invasion. Confocal scanning microscopy of *C. jejuni* 81-176 invasion into single host target cells expressing (A) DN-Rac1, (B) CA-Rac1, (C) DN-Cdc42 or (D) CA-Cdc42. INT-407 cells were transfected with the indicated constructs for 48 hrs, washed and infected for 6 hrs with GFP-expressing 81-176. IF-staining: α-c-Myc antibody for GTPase expression (blue) and rhodamine-phalloidin for F-actin (red). Arrows indicate examples of internalized bacteria. (E) The number of internalized bacteria was quantified in 50 randomly selected blue cells, verifying expression of the respective GTPase construct. Box-Whiskers plots of internalized bacteria per cell are shown with the whiskers giving the minimum and maximum achieved. (***) $P<0.0005$ were considered as statistically significant as compared to the mock control.

In contrast, *C. jejuni* were rarely found internalized into either Rac1-T17N or Cdc42-T17N expressing cells (Fig. 15A and C, respectively). Instead, internalized bacteria were prominently observed in both Rac1-Q61L- and Cdc42-Q61L-expressing INT-407 cells in a statistically significant manner (Fig. 15B and D, respectively). The quantification data are shown in Fig. 15E. Thus, activated Rac1 and Cdc42 positively regulate the uptake of *C. jejuni* in INT-407 epithelial cells.

5.2.4. *Campylobacter jejuni* internalization is accompanied by activation of endogenous Rac1 and Cdc42

The data described above suggest that Rac1 and Cdc42 are involved in *C. jejuni* invasion, but do not show whether these GTPases are activated during infection. To test whether the activation of Rac1 and Cdc42 occurs during infection with *C. jejuni*, GTPase pull-down assays were performed. GTPases cycle between the inactive, GDP-bound and active, GTP-bound forms (reviewed in Schmidt & Hall, 2002). Thus, GTP-loading onto Rac1 and Cdc42 was determined by specific binding of the active GTPase to the Cdc42-Rac1 interactive binding domain of PAK1 fused to glutathione *S*-transferase (GST-CRIB) (Burbelo *et al.,* 1995; Zhao & Manser, 2005). Positive and negative controls were carried out with GTPγ-S and GDP, respectively (data not shown). Monolayers of INT-407 cells were infected with *C. jejuni* for different time periods. Lysates were prepared, and the amount of GTP-loaded Rac1 and Cdc42 was determined. As shown in Fig. 16A-C, prominent activation of Rac1 and Cdc42 in INT-407 cells was detected between 2-4 hrs after infection. These results are consistent with the finding that high rates of *C. jejuni*-induced membrane ruffling and invasion can be detected as soon as 4-6 hrs after infection (Figs. 8-15). Moreover, they further support the hypothesis that activation of both Rac1 and Cdc42 activity is biologically relevant to *C. jejuni* internalization.

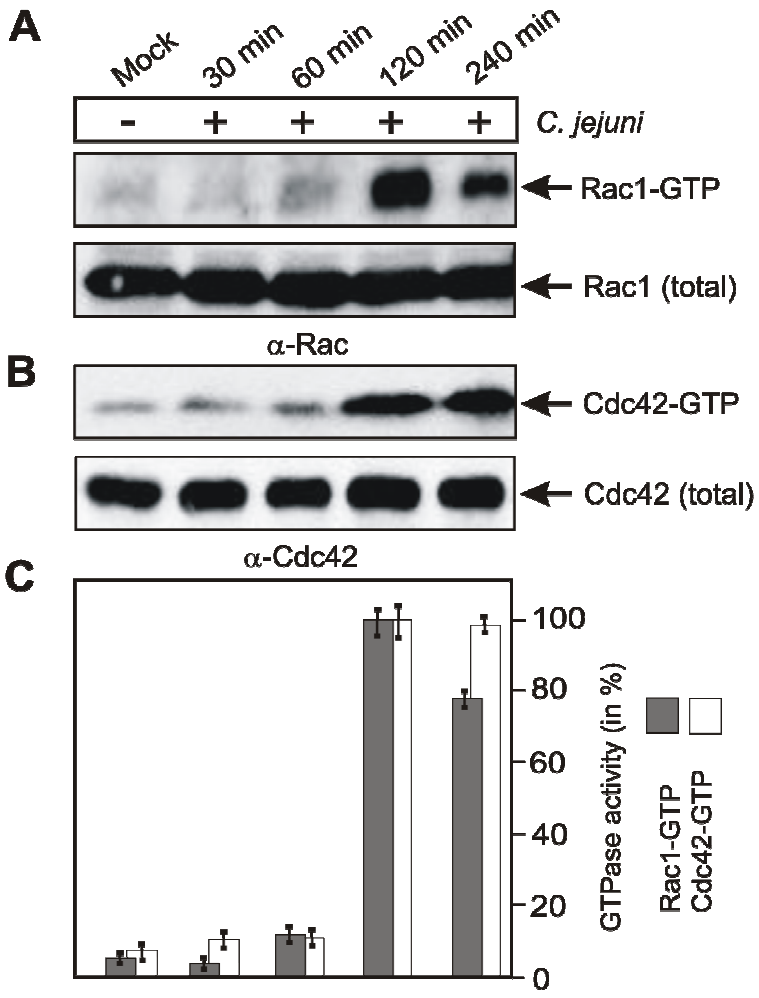


Fig. 16. Time-dependent activation of Rac1 and Cdc42 during *C. jejuni* infection. INT-407 cells were infected with *C. jejuni* for indicated periods of time. The presence of bound, (A) active Rac1-GTP or (B) Cdc42-GTP was analyzed in CRIB-GST pull-down assays followed by Western blotting using α-Rac1 and α-Cdc42 antibodies, respectively. Similar quantities of individual GTPases at every time point were confirmed by Western blotting using equivalent volumes of cell lysates. (C) Quantification of Rac1 and Cdc42 GTPase activities during the course of infection. One hundred % of activity corresponds to the highest amount of detected GTPase-GTP levels.

5.2.5. Activation of Rac1 and Cdc42 by *C. jejuni* involves the fibronectin-binding protein CadF

Next, an attempt was made to identify or exclude bacterial factors involved in *C. jejuni*-induced GTPase activation. For this purpose, five isogenic mutants of *C. jejuni* well-known pathogenicity factors were employed: CadF, KpsS, WaaF, PEB1 and CDT (Table 9). While almost no difference in activation of Rac1 and Cdc42 was observed with the 81-176Δ*cdtB* and 84-25Δ*waaF* mutants, infection with 84-25Δ*kpsS,* and 81-176Δ*PEB1A* mutants slightly enhanced the activation of the GTPases when compared to their respective wt strains (Table 9). Interestingly, the virulence plasmid-free *C. jejuni* strains 84-25 and F38011 (see

Materials & Methods) activated Rac1 and Cdc42 as efficiently as the pVir-carrying strain 81-176, indicating that pVir is not involved in the signaling upstream of GTPases (Table 9). Reduced activation of Rho GTPases was observed in cells infected with the F38011Δ*cadF* mutant strain; this strain less efficiently activated Rac1 (68% compared to wt) and Cdc42 (37% compared to wt) (Fig. 17A-C, Table 9). This further confirmed the findings described in chapter 5.1.4. The activation of Rho GTPases correlated with the association of CadF-positive *C. jejuni* to the cells and their internalization into INT-407 cells, which was significantly higher as compared to the isogenic *cadF* mutant (Fig. 7B, C). Thus, the fibronectin-binding protein CadF plays a role in the activation of Rac1 and Cdc42. However, CadF does not appear to be the sole factor involved in GTPase activation and invasion of *C. jejuni*. The CadF mutant was still able to induce some GTPase activation suggesting that other bacterial factor(s) are also implicated (Fig. 17A-C).

Table 9. Activation of Rac1 and Cdc42 GTPases by *C. jejuni* wild-type strains and isogenic mutants.

***C. jejuni* Strains**	**Function of mutated gene [a]**	**GTPase activation (in %) [b]**		**References**
		Rac1	**Cdc42**	
F38011	wt strain	100 +/-3	100 +/-3	Konkel *et al.,* 1992
F38011Δ*cadF*	fibronectin-binding protein (adhesin)	68 +/-2	37 +/-2	Konkel *et al.,* 1997
84-25	wt strain	100 +/-2	100 +/-3	Blaser *et al.,* 1986
84-25Δ*kpsS*	capsule polysaccharide export protein, involved in biosynthesis of CPS	105 +/-3	125 +/-4	Keo & Blaser, personal communi-cation
84-25Δ*kpsS/kpsS*	insertion mutant complemented with wt *kpsS* gene	102 +/-2	105 +/-4	Keo & Blaser, personal communi-cation
84-25Δ*waaF*	ADP-heptosyltransferase, adds a second heptose to the core oligosaccharide of LOS (loss of function results in truncated LOS)	103 +/-2	101 +/-3	Keo *et al.,* submitted
84-25Δ*waaF/waaF*	insertion mutant complemented with wt *waaF* gene	103 +/-3	101 +/-2	Keo *et al.,* submitted
81-176	wt strain	100 +/-3	100 +/-3	Korlath *et al.,* 1985
81-176Δ*pEB1A*	aspartate/glutamate binding protein of an ABC transporter (adhesin)	99 +/-2	116 +/-2	Misawa & Blaser, 2000
81-176Δ*cdtB*	cytolethal distending toxin (subunit B), exported DNase	101 +/-2	98 +/-2	Hickey *et al.,* 2000

[a] Inactivation of the indicated genes was obtained by insertion of a resistance gene cassette, as published in the given references.
[b] The GTPase activity in infections with wild-type *C. jejuni* was set 100% in each experiment.

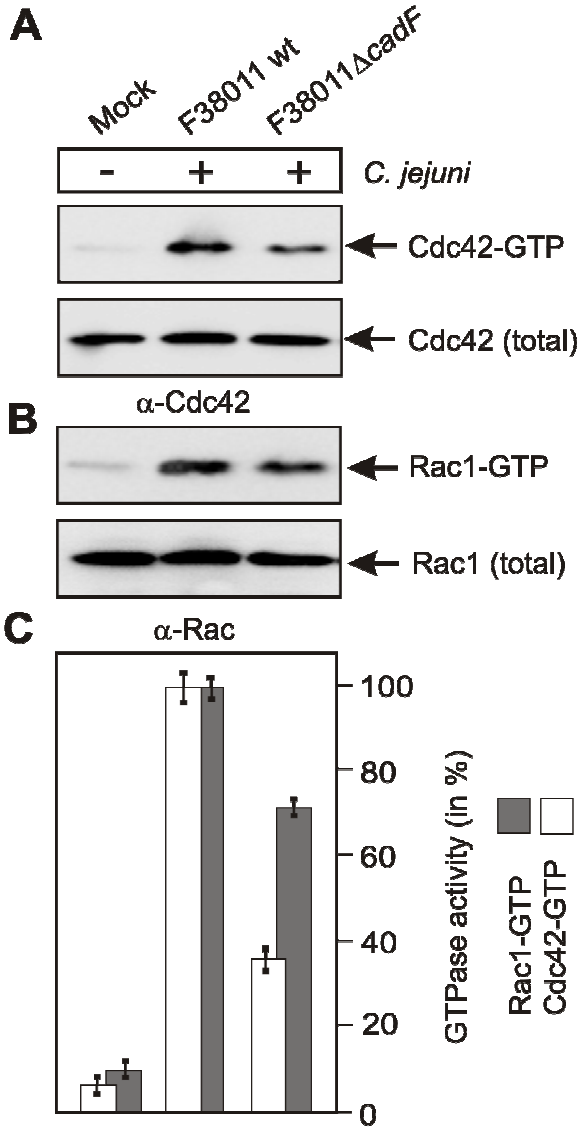


Fig. 17. Importance of CadF expression on *C. jejuni*-induced activation of Rac1 and Cdc42. INT-407 cells were infected with isogenic F38011Δ*cadF* mutant *vs.* wt F38011 for 6 hrs at 37°C. The presence of bound, (A) active Cdc42-GTP or (B) Rac1-GTP was analyzed in CRIB-GST pull-down assays followed by Western blotting using α-Rac1 and α-Cdc42 antibodies, respectively. Similar quantities of individual GTPases in each lane were confirmed by Western blotting using equivalent volumes of cell lysates. (C) Quantification of Rac1 and Cdc42 GTPase activities during the course of infection. One hundred % of activity corresponds to the highest amount of detected GTPase-GTP levels.

5.3. Role of the ß$_1$ integrins, platelet derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR) and the focal adhesion kinase (FAK) in host cell invasion of *Campylobacter jejuni*

5.3.1. ß$_1$ integrins and FAK are required for maximal *C. jejuni* internalization

As the important pathogenicity factor CadF mediates the binding of *C. jejuni* to Fn, the next aim of my study was to investigate whether integrins and FAK are involved in *C. jejuni* uptake. Integrins are a large class of transmembrane receptors composed of heterodimeric α and ß subunits which are critically involved in cell-cell and cell-matrix adhesion (Danen & Yamada, 2001; van der Flier & Sonnenberg, 2001; Hynes, 2002). These surface exposed and structurally conserved proteins are exploited by a number of microbial pathogens to contact and enter host cells and tissues (Hauck, 2002; van der Flier & Sonnenberg, 2001). Many bacteria have evolved surface proteins that bind to integrins or the associated extracellular matrix protein Fn (Schwarz-Linek *et al.,* 2004). Fn recruited to the surface of the bacteria serves as a molecular bridge linking the bacteria with a common host Fn receptor, the integrin $\alpha_5ß_1$ (Joh *et al.,* 1999). To reveal whether the ß$_1$ integrins play a role in the *C. jejuni* internalization, the ß$_1$ integrin-deficient cell line (GD25) (Fässler *et al.,* 1995) and GD25 stably re-expressing mutated integrin subunit ß$_1$A (GD25-ß$_1$A$_{TT788-9AA}$) (Wennerberg *et al.,* 1998), (GD25-ß$_1$A$_{Y783/795F}$) (Wennerberg *et al.,* 2000) and wild-type ß$_1$A (GD25-ß$_1$A) (Wennerberg *et al.,* 1996) were tested by gentamycin protection assay. Invasion of *C. jejuni* was found to be significantly reduced in ß$_1$ integrin-deficient GD25 cells as well as in both mutant cell lines as compared to the GD25-ß$_1$A control ($P \leq 0.001$) (Fig. 18A).

Integrin activation and clustering is associated with tyrosine phosphorylation of the non-receptor kinase FAK, and is a strategy of regulating host signal transduction events leading to actin rearrangements (Tachibana *et al.,* 1995; Miyamoto *et al*., 1998). Indeed, the lowest values of intracellular bacteria were observed with GD25-ß$_1$A$_{Y783/795F}$ cells which are defective in signaling to FAK due to the defect in ß$_1$-dependent autophosphorylation of FAK at tyrosine 397 (Wennerberg *et al.,* 2000) (Fig. 18A). This result suggests that besides ß$_1$ integrin FAK maybe also required for *C. jejuni* uptake. To confirm this idea, mouse fibroblasts derived from FAK-deficient embryos [FAK$^{-/-}$ cells, called here FAK (-)] and FAK (-) cells stably re-expressing HA-epitope-tagged FAK [clone DA2, called here FAK (+)] (Sieg *et al.,* 1999) were employed in gentamicin protection assays. Quantification of intracellular bacteria revealed that FAK-deficient cells were significantly impaired in their ability to internalize *C. jejuni* ($P \leq 0.01$) (Fig. 18C).

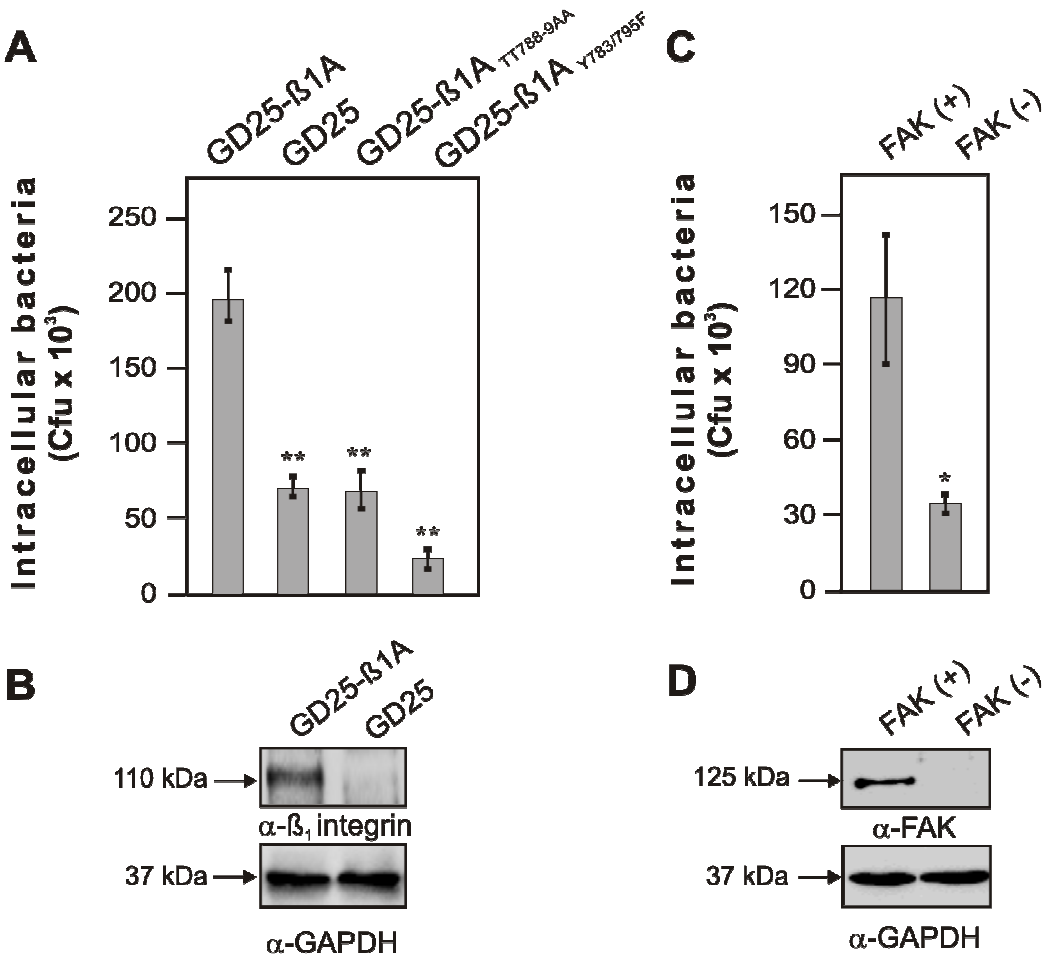


Fig. 18. Effect of ß$_1$ integrin and FAK deficiency on *C. jejuni* invasion. (A) ß$_1$ integrin-deficient cells (GD25) and GD25 stably re-expressing mutated integrin subunit ß$_1$A (GD25-ß$_1$A$_{TT788-9AA}$, GD25-ß$_1$A$_{Y783/795F}$ or wild-type ß$_1$A (GD25-ß$_1$A) cells and (C) FAK-re-expressing [FAK (+)) and FAK-deficient (FAK (-)] cells were infected for 6 hrs with *C. jejuni.* Intracellular *Campylobacter* cells were quantified by gentamicin protection assays. (*) P<0.01 and (**) P≤0.001 were considered as statistically significant. (B) ß$_1$ integrin expression was analyzed by immunoblotting of enriched membrane fractions (Backert *et al.,* 2000) with α-ß$_1$ integrin antibody. (D) FAK expression was analyzed by immunoblotting with α-FAK antibody. GAPDH expression levels were determined as control.

5.3.2. Activation of FAK is critical for efficient uptake of *C. jejuni*

FAK is an important modulator of integrin-dependent focal contacts thereby orchestrating important cellular events such as cell spreading, cell migration or integrin-initiated signaling events (Hauck *et al.,* 2002). To investigate which function of FAK plays a role for the integrin-initiated internalization of *C. jejuni*, FAK (-) cells were transiently transfected with wild-type FAK or different FAK mutants that were either not capable of autophosphorylation (FAK Y397F), were impaired in their kinase activity (FAK K454R), lacked several proline residues necessary for association with SH3-containing proteins such as p130^{CAS} or Graf (FAK Pro$^-$) or the Grb2-binding site (FAK Y925F) (Sieg *et al.,* 1999; Hauck *et al.,* 2002). Gentamicin

protection assays revealed that overexpression of each of these FAK mutants significantly reduced the internalization of *C. jejuni* ($P \leq 0.001$) by about 35-50% (Fig. 19) indicating an important role of FAK signaling in facilitating efficient uptake of *C. jejuni*.

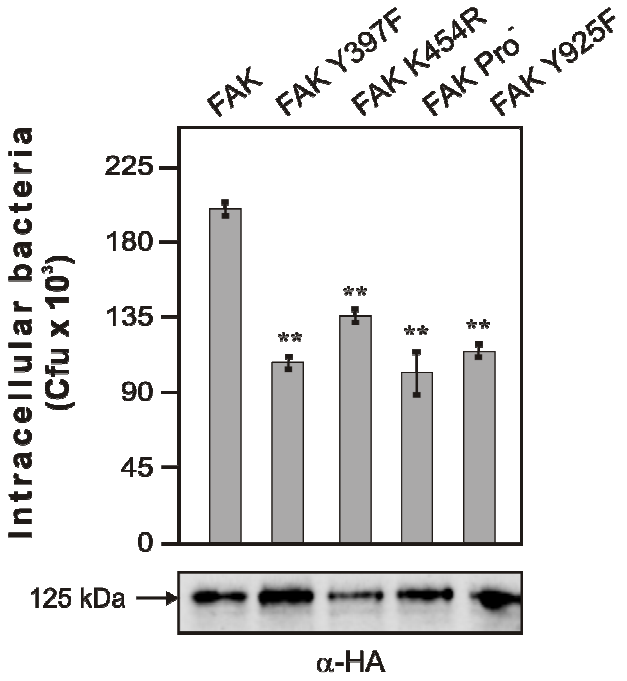


Fig. 19. Interference with FAK function reduces uptake of *C. jejuni*. (A) FAK (-) cells were transfected with the indicated HA-tagged FAK constructs. After 48 hrs, the cells were infected with *C. jejuni* for 6 hrs. (A) Intracellular bacteria were quantified by gentamicin protection assays. (**) $P \leq 0.001$ were considered as statistically significant as compared to wt FAK control. Expression of the individual FAK constructs was verified by Western blot analysis using α-HA antibody.

As outlined above, FAK localizes with β_1-integrins and becomes activated by autophosphorylation at position Y397 in response to many integrin-initiated signaling processes (Parsons, 2003). Consequently, it is not surprising that the most pronounced effect on *C. jejuni* invasion was observed with cells transfected with FAK Y397F mutant. Expression of this mutant resulted in reduction to 53% of wild-type FAK level (Fig. 19). The next experiment was therefore to examine whether *C. jejuni* can stimulate the autophosphorylation of FAK. For this purpose, FAK (+) monolayers were infected with *C. jejuni* wild-type F38011 for different time periods (30 min to 4 hrs) and cell lysates were analyzed by immunoblotting with an antibody specific for FAK phosphorylated at position Y397. To investigate the contribution of the CadF protein for FAK autophosphorylation the effect of the F38011Δ*cadF* mutant was examined in parallel. Infection of FAK (+) cells with *C. jejuni* wild-type F38011 resulted in an increased amount of Y397 phosphorylated FAK, which was detectable within 60 min and remained elevated for at least 4 hrs of incubation (Fig. 20, left lanes). In contrast, FAK phosphorylation was not detected after infection with

F38011Δ*cadF* mutant (Fig. 20, right lanes). These findings suggest that infection with *C. jejuni* leads to activation of FAK by autophosphorylation at position Y397, which is fully dependent on the expression of CadF.

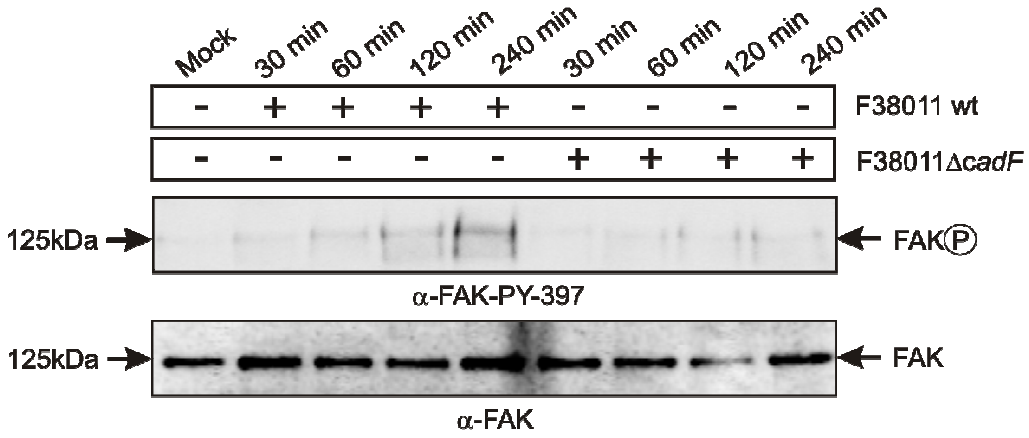


Fig. 20. Activation of FAK upon CadF-mediated cell adhesion and invasion. FAK (+) cells were infected with *C. jejuni* wild-type F38011 *vs.* F38011Δ*cadF* for indicated periods of time. Cell lysates were analyzed by immunoblotting with α-FAK-PY-397. Total FAK expression levels were determined as control.

5.3.3. Importance of FAK and CadF expression for *C. jejuni*-induced Rac1 and Cdc42 activation

The results described above suggest that both FAK and *Campylobacter* Fn-binding protein CadF are important for *C. jejuni* internalization. Interestingly, the time-dependent activation of Rho GTPases correlated with the association and internalization of CadF-positive *C. jejuni* to the cells (see Fig. 7 in chapter 5.1.4 and Fig. 17 in chapter 5.2.5). This led me to test the role of FAK in CadF-mediated Rac1 and Cdc42 activation. For this purpose, monolayers of FAK (+) and FAK (-) cells were infected with *C. jejuni* isogenic F38011Δ*cadF* mutant *vs.* wt F38011 for different time periods and CRIB-GST pull-down assays were performed. While activated Rac1 and Cdc42 were detected in FAK (+) cells 4 hrs after infection, no detectable activation was found in FAK (-) cells during the course of infection (Fig. 21A-E), indicating the involvement of FAK in signaling upstream of Rac1 and Cdc42 activation during *C. jejuni* invasion. Furthermore, reduced activation of Rho GTPases was observed in FAK (+) cells infected with the F38011Δ*cadF* mutant strain (Fig. 21A-C). This result is consistent with the finding that F38011Δ*cadF* less efficiently activated Rac1 and Cdc42 in INT-407 cells (Fig. 17A-C and Table 9). Moreover, these findings further support the hypothesis that the CadF protein plays a role in signaling leading to the activation of Rac1 and Cdc42. However, as observed in INT-407 cells, the CadF mutant was still able to induce some GTPase activation in Fak (+) cells suggesting that other bacterial factor(s) are also implicated in this signaling (Fig. 21A-C).

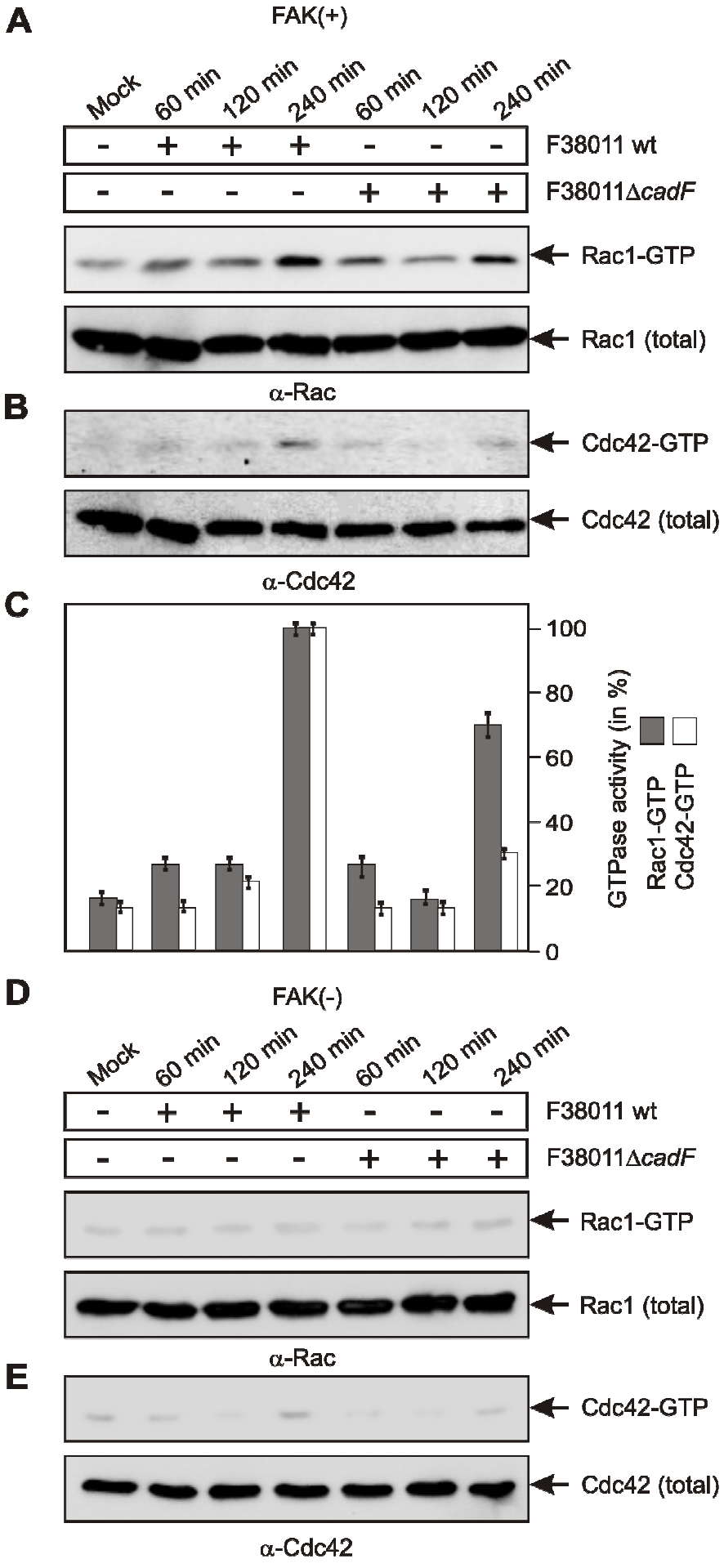

A
FAK(+)
Mock
60 min
120 min
240 min
60 min
120 min
240 min
F38011 wt
F38011ΔcadF
Rac1-GTP
Rac1 (total)
α-Rac
B
Cdc42-GTP
Cdc42 (total)
α-Cdc42
C
100
80
60
40
20
0
GTPase activity (in %)
Rac1-GTP
Cdc42-GTP
D
FAK(-)
Mock
60 min
120 min
240 min
60 min
120 min
240 min
F38011 wt
F38011ΔcadF
Rac1-GTP
Rac1 (total)
α-Rac
E
Cdc42-GTP
Cdc42 (total)
α-Cdc42

Fig. 21. Refers to the figure on page 72. Importance of FAK and CadF expression for *C. jejuni*-induced activation of Rac1 and Cdc42. (A, B, C) FAK (+) and (C, D) FAK (-) cells were infected with *C. jejuni* wt F38011 *vs.* isogenic F38011Δ*cadF* mutant for indicated periods of time. The presence of bound, (A, D) active Rac1-GTP or (B, E) Cdc42-GTP was analyzed in CRIB-GST pull-down assays followed by Western blotting using α-Rac1 and α-Cdc42 antibodies, respectively. Similar quantities of individual GTPases at every time point were confirmed by Western blotting using equivalent volumes of cell lysates. (C) Quantification of Rac1 and Cdc42 GTPase activities during the course of infection of FAK (+) cells. One hundred % of activity corresponds to the highest amount of detected GTPase-GTP levels.

5.3.4. Activation of Rac1 and Cdc42 by *C. jejuni* involves another bacterial component: flagellar apparatus

Since CadF is not the sole bacterial factor involved in *C. jejuni*-induced GTPase activation, the next aim was to search for other factor(s) playing a role in this signaling. The flagellar apparatus was reported to be one of the most intensively investigated pathogenicity determinant in *Campylobacter*. FlaA/B proteins have been shown to be required for bacterial colonization in a number of animal models (Morooka *et al.,* 1985; Wassenaar *et al.,* 1993; Hendrixson & DiRita, 2004), and they play an active but yet unknown role in the invasion of epithelial cells (Wassenaar *et al.,* 1991; Grant *et al.,* 1993; Yao *et al.,* 1994). To test a potential role in GTPase activation, FAK (+) cells were infected with *C. jejuni* wt 81-176, flagellin mutant 81-176Δ*flaA/B* and flagellar biosynthesis mutant 81-176Δ*flhA*. As expected activated Rac1 and Cdc42 were detected in FAK (+) cells between 2-4 hrs after infection with wt 81-176 (Fig. 22A-C), confirming the findings obtained with INT-407 cells (Fig. 16A-C). However, no detectable activation was found in FAK (+) cells infected with 81-176Δ*flaA/B* or 81-176Δ*flhA* during the course of infection (Fig. 22A-C), indicating important role of flagellar apparatus in activation of Rac1 and Cdc42 by *C. jejuni*. Taken together, the findings described here suggest that both the flagella and CadF are able to trigger GTPase signaling in infected host cells.

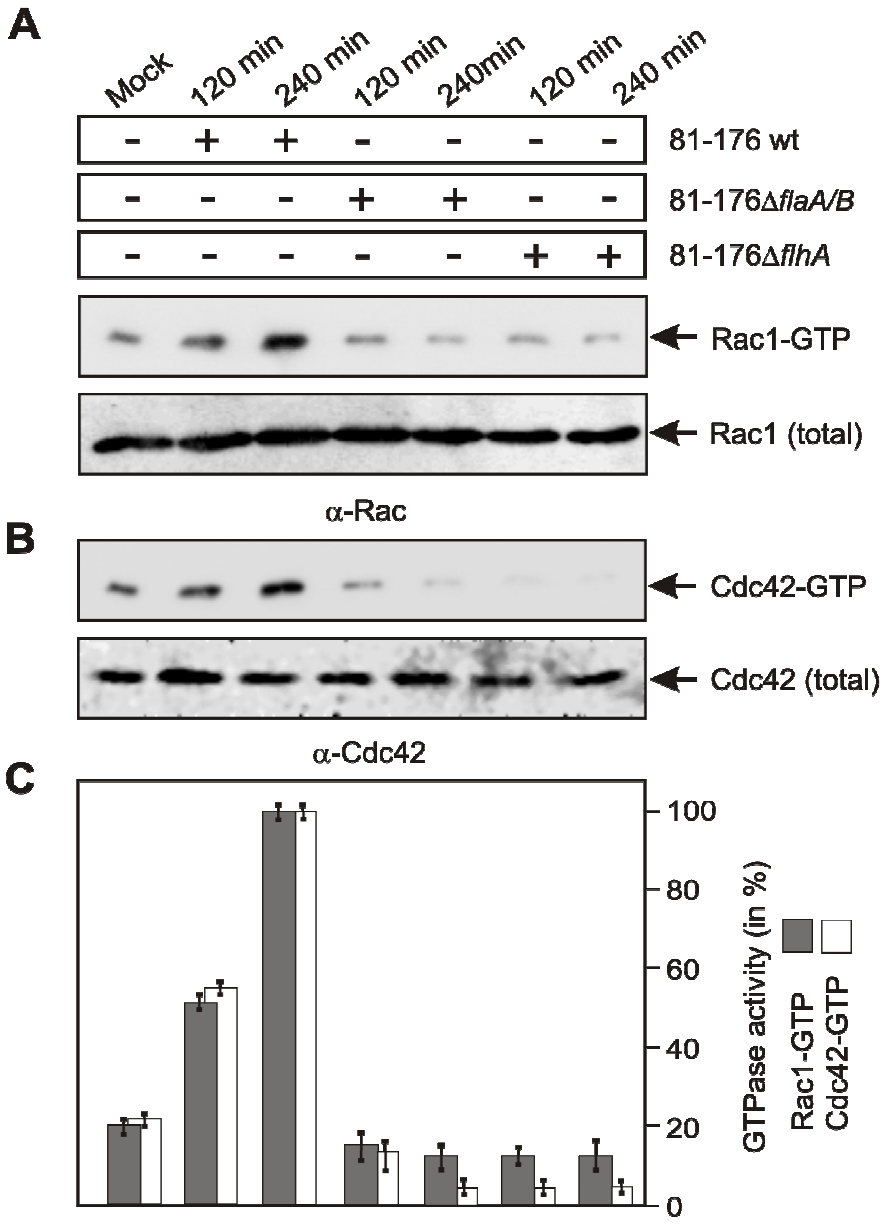


Fig. 22. Importance of FAK and flagellar apparatus for *C. jejuni*-induced activation of Rac1 and Cdc42. FAK (+) cells were infected with *C. jejuni* wt 81-176 *vs* 81-176Δ*flaA/B* and 81-176Δ*flhA* mutants for indicated periods of time. The presence of bound, (A) active Rac1-GTP or (B) Cdc42-GTP was analyzed in CRIB-GST pull-down assays followed by Western blotting using α-Rac1 and α-Cdc42 antibodies, respectively. Similar quantities of individual GTPases at every time point were confirmed by Western blotting using equivalent volumes of cell lysates. (C) Quantification of Rac1 and Cdc42 GTPase activities during the course of infection. One hundred % of activity corresponds to the highest amount of detected GTPase-GTP levels.

5.3.5. Identification of guanine nucleotide exchange factors involved in *C. jejuni*-induced activation of Rac1 and Cdc42 and host cell invasion

Next aim was to determine a number of signaling components upstream of GTPases activation during infection with *C. jejuni.* Cycling of Rho GTPases between the inactive and active forms is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs stimulate the exchange of GDP for GTP to generate the active form of GTPase, which is then capable of recognizing downstream targets, or effector proteins (reviewed in Schmidt & Hall, 2002). Whereas some of the GEFs display specific activity toward one Rho GTPase, *e.g.* Tiam1 or DOCK180 for Rac1 (Michiels *et al.,* 1995; 1997; Kiyokawa *et al.,* 1998; Cote & Vuori, 2002; 2007), others can activate multiple Rho GTPases promiscuously, *e.g.* Vav-2 can activate RhoA, Cdc42 and Rac1 (Abe *et al.,* 2000), α-PIX activates Rac1 and Cdc42 (Manser *et al.,* 1998; Yoshii *et al.,* 1999), and Trio can activate Rac1 and RhoA (Debant *et al.,* 1996). To identify which GEFs are involved in *C. jejuni* invasion, the expression of Vav-2, DOCK180, α-PIX, Tiam1 or Trio was down-regulated using target-specific siRNA. While down-regulation of Vav-2, DOCK180, α-PIX, and Tiam1 led to the significant reduction in *C. jejuni* internalization (Fig. 23A-D), both down-regulation of Trio (Fig. 23E), and transfection with non-targeting scrambled control sequence had no effect on *C. jejuni* uptake as quantified by gentamicin protection assays (Fig. 23A-E). It has to be noted that down-regulation of these individual GEFs did not lead to a complete blockade of *C. jejuni* uptake. This suggests that multiple GEFs such as Vav-2, DOCK180, α-PIX, and Tiam1 but not Trio may play a role in *C. jejuni* invasion. Although not shown by direct approach, these GEFs could be involved in *C. jejuni*-induced Rac1 and Cdc42 activation.

The role of Tiam1 for *C. jejuni* internalization was further examined with the use of a specific Rac1 inhibitor, NSC23766 (Gao *et al.,* 2004). This cell-permeable compound specifically inhibits Rac1 GDP/GTP exchange activity by interfering with the interaction between Rac1 and its GEFs Tiam1 and Trio. Furthermore, it exhibits no effect on Cdc42 or RhoA activation and does not affect Rac1 interaction with its effector protein p21 activated kinase 1 (PAK1) (Gao *et al*., 2004). Gentamycin protection assay revealed that the uptake of *C. jejuni* was significantly reduced in the presence of the NSC23766, a result which further confirms a role of Rac1 and Tiam1 for *C. jejuni* invasion (Fig. 24).

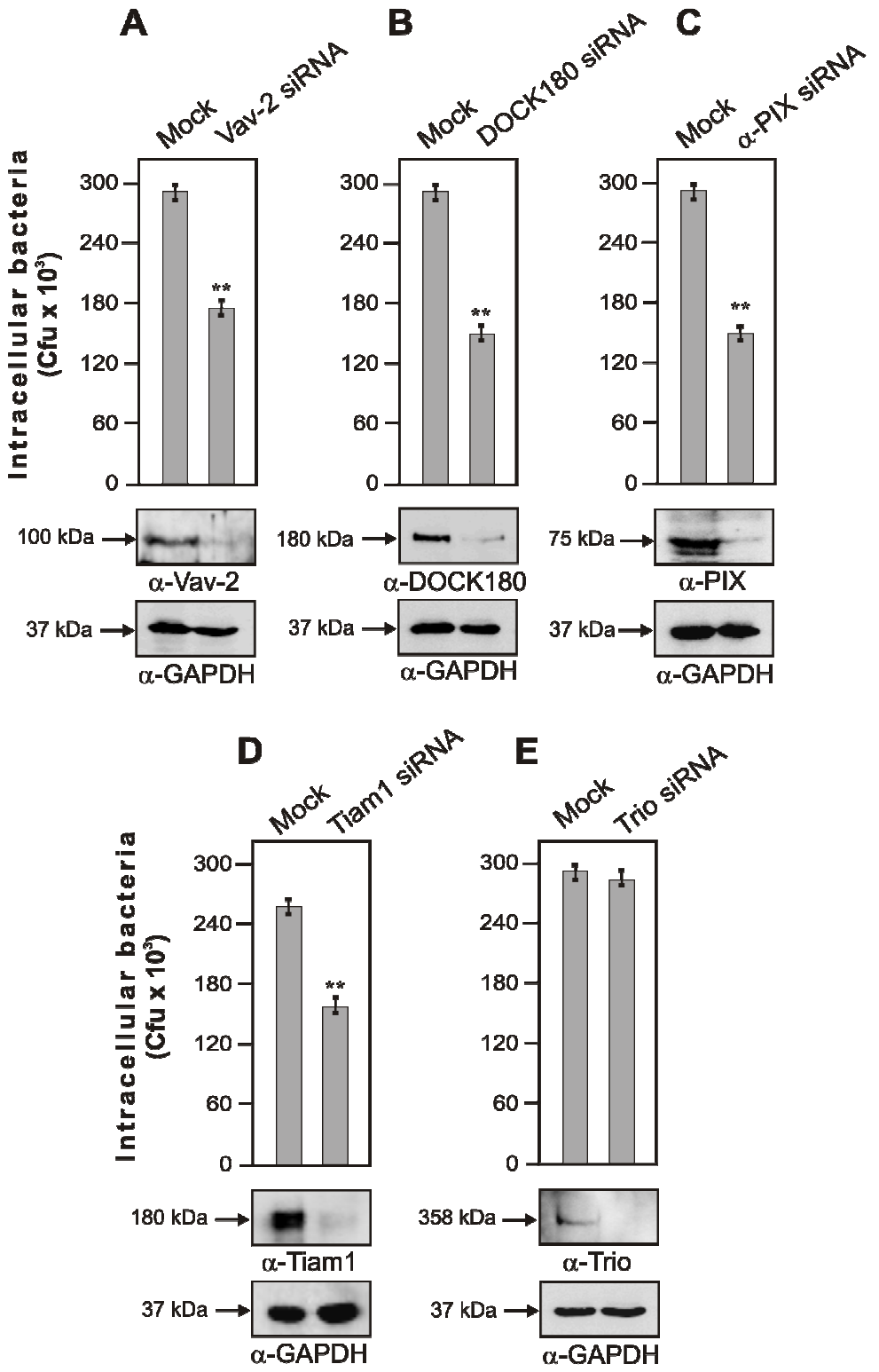


Fig. 23. Effect of down-regulation of GEFs for Rac1 and Cdc42 on *C. jejuni* invasion. INT-407 cells were transfected with small interfering RNA (siRNA) for (A) Vav-2 (B) DOCK180 (C) α-PIX (D) Tiam1 (E) Trio. After 48 hrs, cells were infected with *C. jejuni* for 6 hrs. Intracellular bacteria were quantified by gentamicin protection assays. (**) $P \leq 0.001$ were considered as statistically significant as compared to the mock control. Immunoblotting with indicated antibodies confirmed knockdown of the proteins. To detect Tiam1 immunoprecipitation with α-Tiam1 antibody was performed. GAPDH expression levels were determined as control.

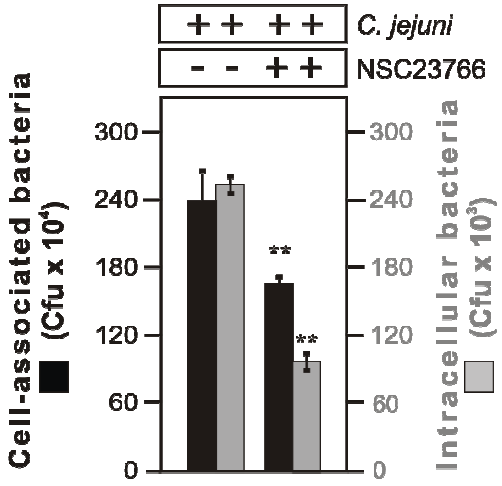


Fig. 24. Effect of the Rac1 inhibitor NSC23766 on *C. jejuni* invasion. INT-407 monolayers were pre-incubated for 30 min with 50 µM NSC23766 and infected with *C. jejuni* for 6 hrs. Total cell associated and intracellular *Campylobacter* cells were quantified by gentamicin protection assays. (**) $P \leq 0.001$ were considered as statistically significant as compared to the mock control.

5.3.6. Identification of upstream kinases involved in *C. jejuni*-induced activation of Rac1 and Cdc42 and host cell invasion

To further elucidate signaling pathways leading to *C. jejuni*-induced activation of Rac1 and Cdc42, several well-known pharmacological kinase inhibitors were applied. In previous studies, treatment of the cells with kinase inhibitors resulted in a decrease of *C. jejuni* invasion (Wooldridge *et al.,* 1996; Biswas *et al.,* 2004; Hu *et al.,* 2006a). In agreement with these results, invasion of *C. jejuni* was significantly reduced in the presence of the kinase inhibitors genistein, tyrphostin-46, wortmannin, or staurosporine (Fig. 25A). To test if the latter effect is based on inhibition of Rac1 and Cdc42 activation, INT-407 cells pre-treated with the inhibitor were infected with *C. jejuni* for 2 hrs. Rac1 and Cdc42 activity was then assessed by CRIB-pull-down assay as described above. Genistein, a broad-spectrum inhibitor of tyrosine kinases, had no significant influence on the activation of Rac1-GTP (Fig. 25B, D) but it reduced the levels of Cdc42-GTP (Fig. 25C, D). Furthermore, tyrphostin-46, an inhibitor of EGFR, $p56^{Lck}$ and PDGFR, and wortmannin, an inhibitor of PI3 kinase, completely blocked the activation of Cdc42 but not Rac1 during infection with *C. jejuni* (Fig. 25). Thus, the genistein-/tyrphostin-/wortmannin-mediated decrease in bacterial cell entry may be based on reduced Cdc42 activation. Staurosporine, a potent broad-spectrum inhibitor of serine/threonine kinases, that also reduced bacterial uptake, completely inhibited the activation of both Rac1 and Cdc42, which may account for the reduced bacterial uptake. Although the pleiotropic effects of the inhibitors cannot be fully ruled out, these data suggest that the EGFR, PDGFR, p56 Lck, PI3 kinase, and several tyrosine kinases are involved in *C. jejuni*-induced activation of Cdc42 but not Rac1, while serine/threonine kinases appear to be involved in the activation of both Rac1 and Cdc42.

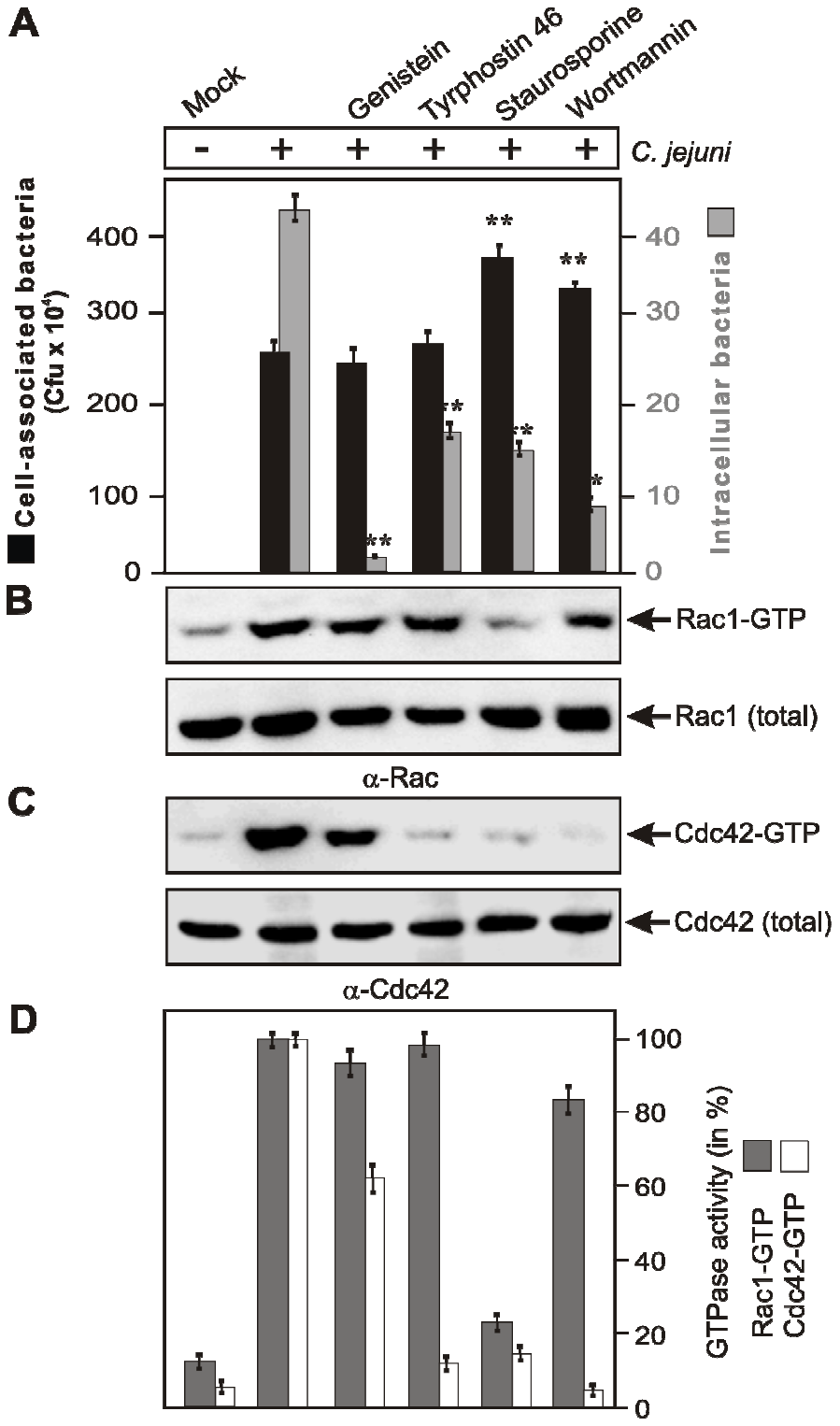


Fig. 25. Effects of pharmacological inhibitors on the activation of small Rho GTPases by *C. jejuni*. INT-407 monolayers were pre-incubated for 30 min with the indicated inhibitors and infected with *C. jejuni* for 6 hrs. (A) Total cell associated and intracellular *Campylobacter* cells were quantified by gentamicin protection assays, (**) $P \leq 0.001$. The presence of bound, (B) active Rac1-GTP or (C) Cdc42-GTP was analyzed in CRIB-GST pull-down assays followed by Western blotting using α-Rac1 and α-Cdc42 antibodies, respectively. Similar quantities of individual GTPases in each lane were confirmed by Western blotting using equivalent volumes of cell lysates. (D) Quantification of Rac1 and Cdc42 GTPase activities during the course of infection. One hundred % of activity corresponds to the highest amount of detected GTPase-GTP levels.

5.3.7. Vav-2 links receptor tyrosine kinases (RTKs) to Rac1 and Cdc42 activation during infection with *C. jejuni*

The inhibitor studies described above indicated a potential role of PDGFR and EGFR both in *C. jejuni*-induced GTPases activation and host cell invasion. To further corroborate these findings, INT-407 cells were transiently transfected with wild-type PDGFR and EGFR constructs, and their respective dominant-negative (DN) forms, followed by infection with *C. jejuni* for 6 hrs. Gentamicin protection assays showed that overexpression of either DN mutant significantly reduced the internalization of *C. jejuni*, further suggesting the involvement of PDGFR and EGFR in uptake of *C. jejuni* (Fig. 26). Notably, transfection with both DN-PDGFR and DN-EGFR constructs resulted in no additive reduction of *C. jejuni* invasion. The latter finding suggests that besides EGFR and PDGFR other signaling pathway(s) are also implicated in *C. jejuni* internalization.

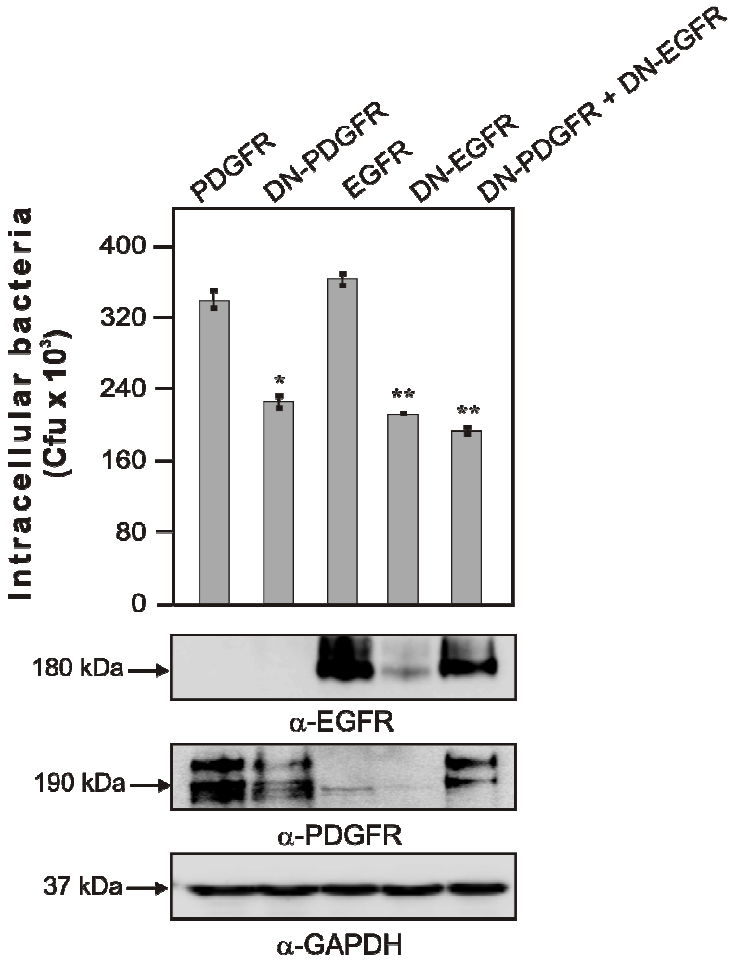


Fig. 26. Effect of overexpression of dominant-negative forms of PDGFR and EGFR on *C. jejuni* uptake. 48 hrs post transfection INT-407 cells were infected with *C. jejuni* for 6 hrs. Intracellular bacteria were quantified by gentamicin protection assays. (*) $P \leq 0.01$ and (**) $P \leq 0.001$ were considered as statistically significant as compared to the wt control. Expression of the individual constructs was verified by Western blot analysis using α-EGFR and α-PDGFR antibodies. GAPDH expression levels were determined as control.

Vav-2 is a substrate for EGFR/PDGFR and Rac1/Cdc42 can be activated downstream of both receptors through Vav-2 exchange activity (Liu & Burridge, 2000; Marcoux & Vuori, 2003; Tamas *et al.,* 2003). As siRNA mediated gene silencing of Vav-2 interfered with uptake of *C. jejuni,* the impact of Vav-2 on *C. jejuni* cell entry was further examined. For this purpose, INT-407 cells were transiently transfected with the wild-type Vav-2 and different Vav-2 mutants that were either impaired in EGFR-dependent phosphorylation of Vav-2 (Vav-2 Y172/159F), lacked the primary phosphatidylinositol 3,4,5-triphosphate binding site (Vav-2 R425C) or were not capable of binding to activated EGF receptor (Vav-2 W673R and Vav-2 G693R) (Tamas *et al.,* 2003). Gentamicin protection assays revealed that overexpression of either Vav-2 mutant significantly reduced the internalization of *C. jejuni* ($P \leq 0.001$) (Fig. 27A), confirming the role of Vav-2 in uptake of *C. jejuni*. This result was further verified by transfection of INT-407 cells with dominant-negative form of Vav-2 which also significantly reduced *C. jejuni* internalization ($P=0.007$) (Fig. 27B). Therefore it is likely that Vav-2 may contribute to the *C. jejuni* induced EGFR/PDGFR-dependent Rac1 and Cdc42 activation.

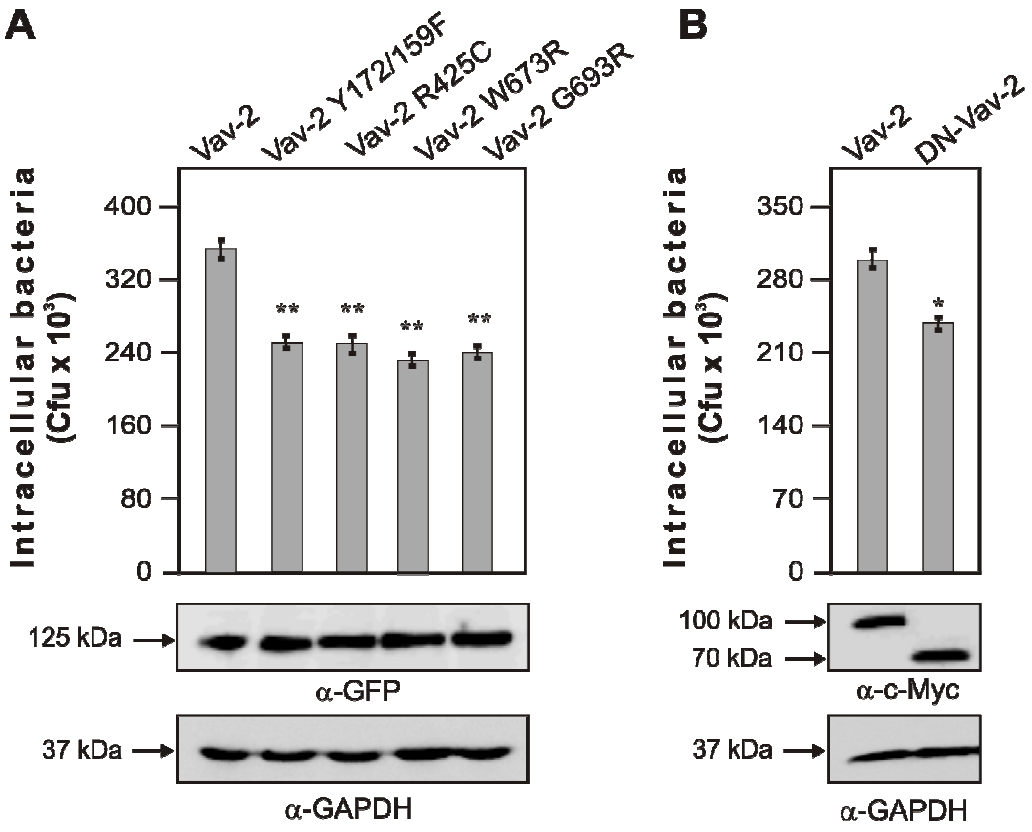


Fig. 27. Interference with Vav-2 function reduces the uptake of *C. jejuni* in host cells. INT-407 cells were transfected with indicated (A) GFP-tagged and (B) Myc-tagged Vav-2 constructs. After 48 hrs, cells were infected with *C. jejuni* for 6 hrs. Intracellular bacteria were quantified by gentamicin protection assays. (*) $P \leq 0.01$ and (**) $P \leq 0.001$ were considered as statistically significant as compared to wt Vav-2 control. (B) Expression of the individual Vav-2 constructs was verified by Western blot analysis using α-GFP or α-c-Myc antibodies. GAPDH expression levels were determined as control.

5.4. Role of the surface array protein SapA in infection of host cells with *Campylobacter fetus*

5.4.1. Identification of a 97 kDa phospho-protein during infection with *C. fetus*

Since the tyrosine phosphorylation of proteins has a central role during signal transduction in eukaryotes (Blume-Jensen & Hunter, 2001; Pawson, 2004), the tyrosine phosphorylation patterns of host cells infected with *Campylobacter* were investigated. Western blot analysis with phospho-specific antibody α-PY-99 revealed strain-dependent 97 kDa phospho-protein for *C. fetus* 97-3574 strain. It was not observed for all *C. jejuni* strains and *C. fetus* 5361 strain (Fig. 28A). To identify the 97 kDa protein, the band was excised from Coomassie-stained SDS-PAGE gel and subjected to matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) which was performed by cooperation partner Dr. Manfred Nimtz (Helmholtz Center for Infection Research, Braunschweig, Germany). By this approach the 97 kDa protein was identified as the bacterial surface array protein SapA of *C. fetus* (accession no. P35827). Nine SapA peptides were detected, leading to sequence coverage of 41% (Fig. 28B, C). However, the MALDI-MS analysis did not identify any phospho-peptides of SapA. These findings were very surprising because SapA is not an effector protein of *C. fetus* but a well known surface array protein (Blaser & Pei, 1993; Blaser, 1998).

5.4.2. Cloning, overexpression and purification of *C. fetus* SapA

To investigate the role of SapA during infection, *sapA* gene was amplified and two vectors, pRK5-*sapA* and pGEX-4T-1-*sapA*, were constructed. Subsequently, SapA was overexpressed and purified as glutathione S-transferase (GST)-fusion protein with use of glutathione-affinity column, followed by gel filtration through a HiLoad 16/60 Sephacryl S-200 HR gel filtration column, as depicted in Materials & Methods. Using described procedures, a 500 ml bacterial culture yielded 430 µg of protein in main fraction and 880 µg of protein of side fraction, of which SapA was the predominant protein species. Fractions from each purification step were subjected to SDS-PAGE/Coomassie staining analysis. On basis of that, SapA-GST purified *via* gel filtration column was judged to be of >95% homogeneity (Figure 29). Unfortunately, cleavage of SapA from GST with thrombin, even after many optimizing trials, was not very efficient. Hence, SapA-GST was used in most experiments with purified GST alone as control. Pure SapA without GST tag was utilized only when indicated. Purified SapA-GST was used for generation of α-SapA antibody (Materials & Methods).

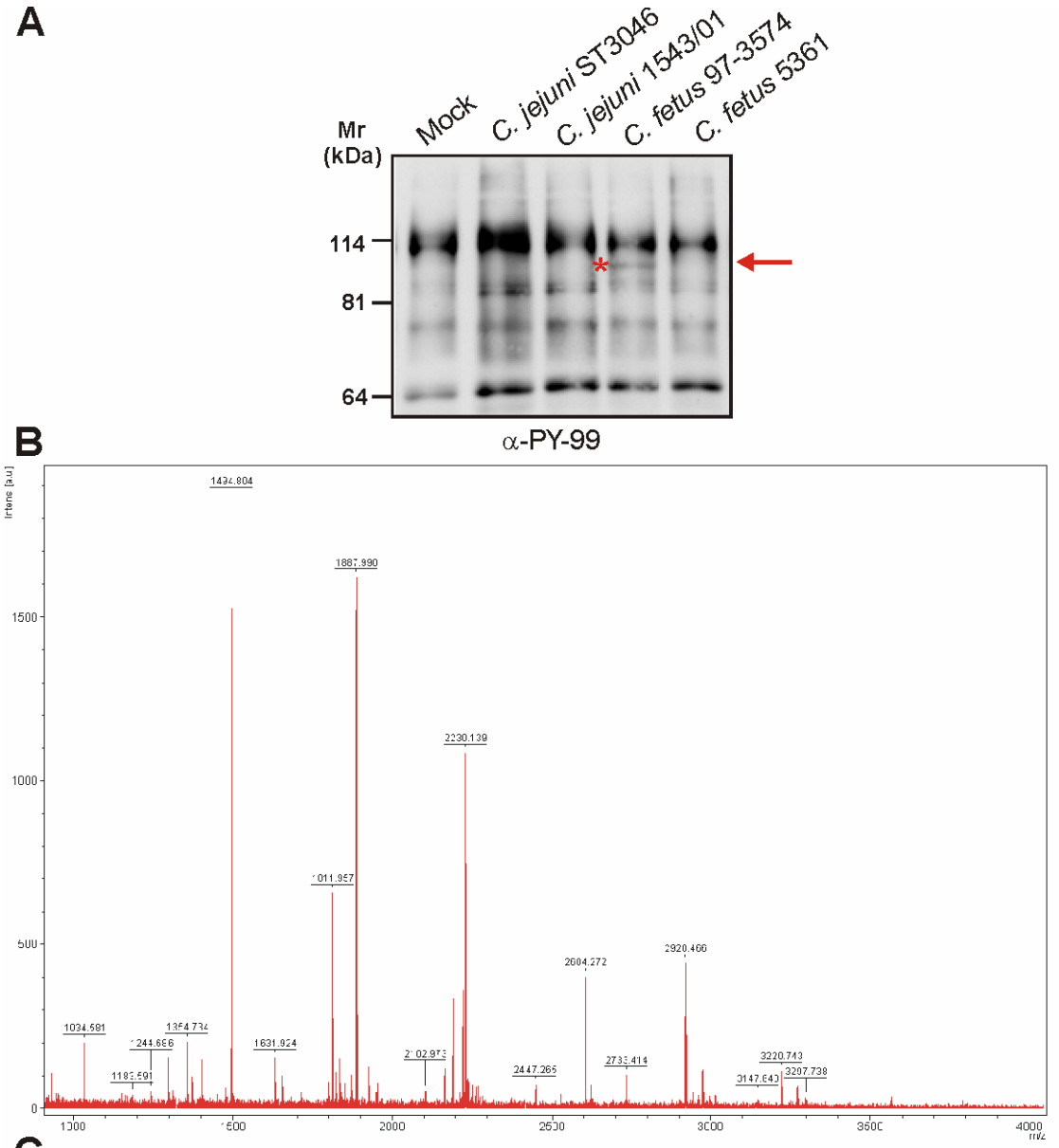


Fig. 28. Identification of a 97 kDa phospho-protein by immunoblotting and MALDI-MS. (A) HeLa cells were infected with indicated *Campylobacter* strains for 24 hrs. Cell lysates were analyzed by immunoblotting with α-PY-99 antibody. Asterisk shows additional 97 kDa phospho-protein band which is absent in other lysates (B) MALDI-MS spectrum of peptide pattern obtained after digestion of 97 kDa protein excised from Coomassie-stained SDS-PAGE gel with trypsin. (C) Identified peptides highlighted in red were matched against NCBI protein database and were identified as part of shown *C. fetus* surface array protein SapA.

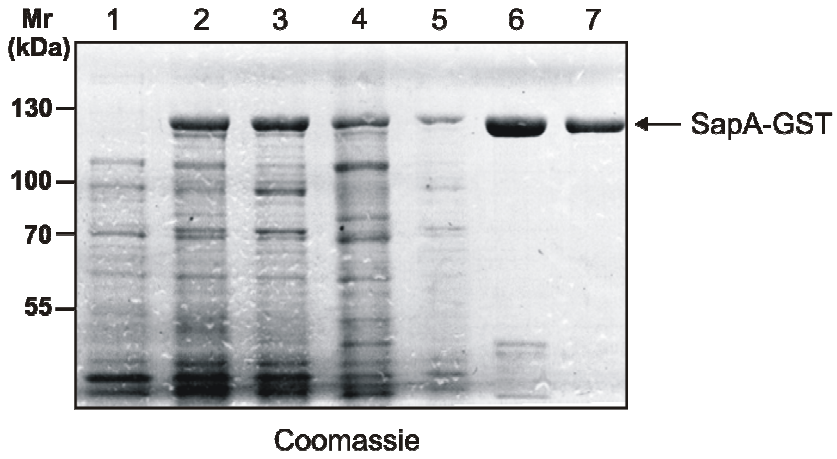


Fig. 29. Overexpression and purification of SapA. Coomassie-stained SDS-PAGE gel of *E. coli* BL21-pGEX-4T-1-*sapA* whole cell extract (1) before and (2) after IPTG induction, (3) supernatant (or soluble) fraction of the cell lysate after induction, (4) pellet (or insoluble) fraction of the cell lysate after induction, (5) flow-through from glutathione-affinity column, (6) eluate from glutathione-affinity column (peak fraction) and (7) purified SapA-GST from gel filtration.

To further confirm that the identified 97 kDa phospho-protein is SapA and to verify the specificity of the generated α-SapA antibody, Western blot analysis of cell lysates after *Campylobacter* infection was performed with α-PY-99 and α-SapA antibodies. The 97 kDa protein band was detected both with α-PY-99 and α-SapA for *C. fetus* 97-3574 strain as expected (Fig. 30A, B). In agreement with these findings, no SapA bands and no 97 kDa phospho-proteins were observed with *C. jejuni* ST 3046, 1543/01 and *C. fetus* 5361 (Fig. 30). These observations suggest that SapA from *C. fetus* strain 97-3574 undergoes tyrosine phosphorylation during infection.

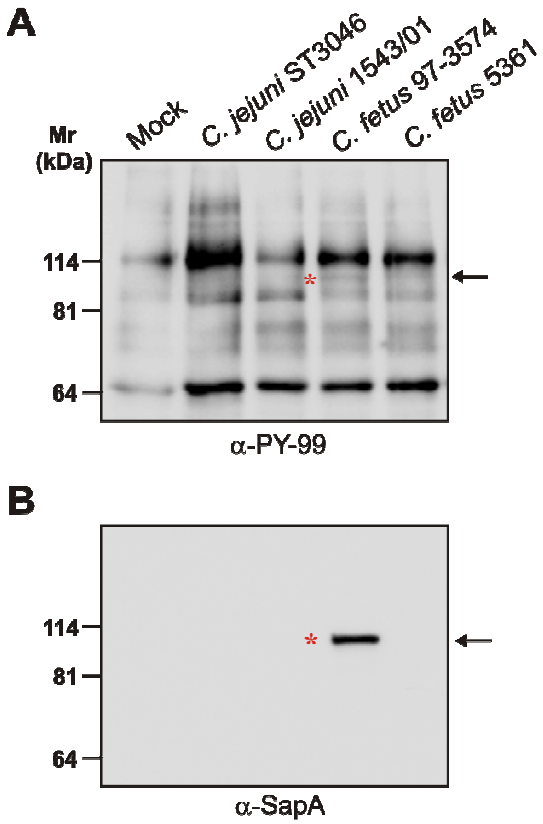


Fig. 30. Identification of a 97 kDa phospho-protein as SapA. HeLa cells were infected with indicated *C. jejuni* and *C. fetus* strains for 24 hrs. Cell lysates were analyzed by immunoblotting with (A) α-PY-99 antibody followed by stripping and re-probing with (B) α-SapA antibody. Asterisk shows the 97 kDa SapA protein band.

5.4.3. SapA is phosphorylated by Src kinase *in vitro* and *in vivo*

To prove whether SapA can be phosphorylated by well-known host cell tyrosine kinases, *in vitro* kinase assays were performed using purified SapA-GST and recombinant c-Src kinase or c-Abl kinase. Whereas strong SapA phosphorylation signals were detected after co-incubation of SapA-GST with recombinant c-Src, only a weak signal was observed with c-Abl. As control, reaction without recombinant kinases was unable to phosphorylate SapA (Fig. 31A). This indicates that both kinases can phosphorylate SapA *in vitro*, but c-Src phosphorylates SapA more efficiently than c-Abl. To confirm that SapA can function as a c-Src substrate, purified SapA without GST was tested in *in vitro* kinase assay with recombinant c-Src kinase. As expected, incubation of c-Src with SapA resulted in SapA phosphorylation (Fig. 31B). These findings demonstrate that SapA serves as c-Src substrate *in vitro*.

To directly prove that SapA can be phosphorylated in cultured host cells, SapA was transiently expressed in INT-407 cells and immunoprecipitated with the α-SapA antibody. Subsequently, phosphorylation of SapA was analyzed by immunoblotting with α-PY-99. As expected, a phosphorylated band of 97 kDa was observed in INT-407 cells expressing

Myc-tagged SapA but not in cells expressing the empty vector control. The identity of the band as SapA was verified both with α-SapA and α-c-Myc antibodies (Fig. 31C). To determine whether Src kinase is important for SapA phosphorylation in cultured cells *in vivo*, infection was performed with *C. fetus* S2+ strain in presence or absence of PP2, a Src-specific tyrosine kinase inhibitor (Hanke *et al.,* 1996), followed by immunoprecipitation of SapA with α-SapA antibody and Western blot analysis with α-PY-99. Treatment of the infected cells with PP2 blocked SapA phosphorylation (Fig. 31D), confirming that SapA can be phosphorylated during *C. fetus* infection of cultured cells by Src kinase *in vivo*.

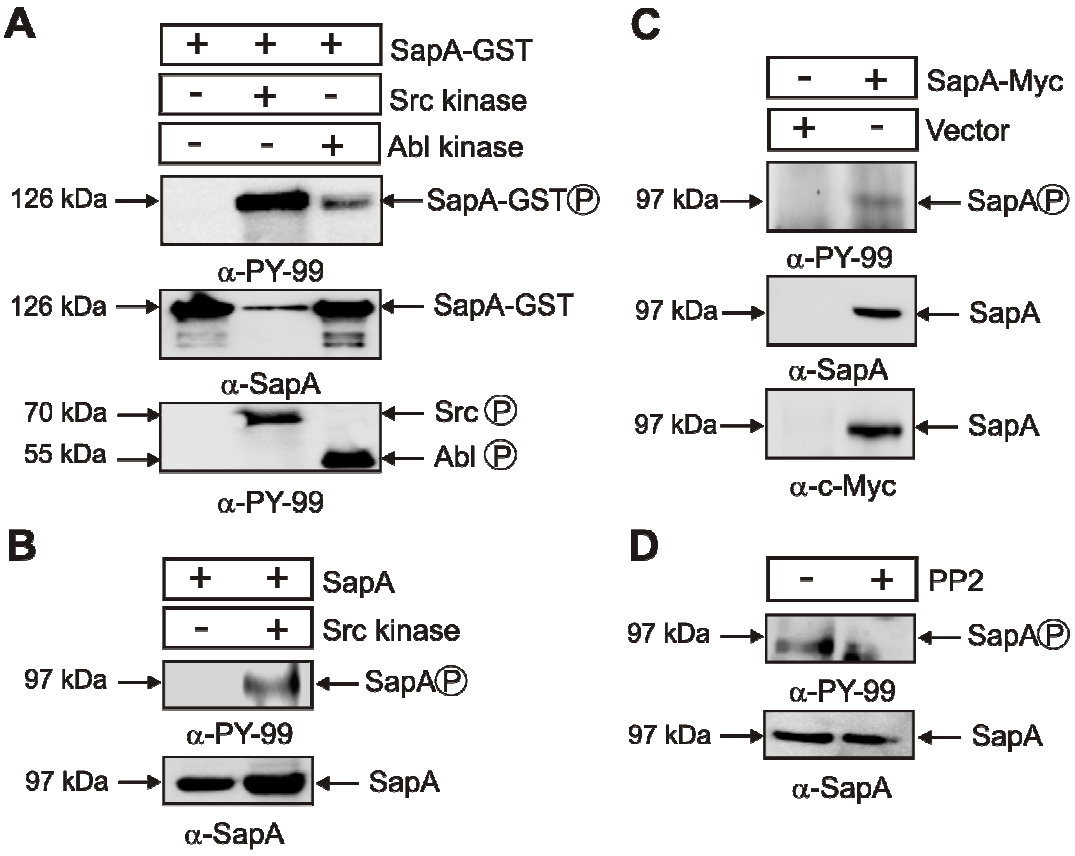


Fig. 31. Tyrosine phosphorylation of SapA *in vivo* and *in vitro*. (A) *In vitro* kinase assay with SapA-GST co-incubated with recombinant c-Src or c-Abl followed by immunoblotting with α-PY-99 (upper panel), stripping and re-probing with α-SapA (middle panel). As positive control, autophosphorylation of both kinases is shown (lower panel). (B) *In vitro* kinase assay with pure SapA without GST co-incubated with recombinant c-Src followed by immunoblotting with α-PY-99 (upper panel), stripping and re-probing with α-SapA (lower panel). (C) SapA phosphorylation in cultured cells. INT-407 cells were transfected with a vector encoding Myc-tagged SapA and empty vector as control. Subsequently, immunoprecipitation with α-SapA antibody was performed and SapA phosphorylation was analyzed by immunoblotting with α-PY-99 (upper panel). Expression of SapA was determined with α-SapA and α-c-Myc antibodies (middle and lower panels, respectively). (D) Phosphorylation of SapA during *C. fetus* infection. THP-1 cells were infected for 6 hrs with *C. fetus* S2+ strain in absence or presence of PP2 inhibitor. Subsequently, immunoprecipitation with α-SapA antibody was performed, followed by immunoblotting with α-PY-99 (upper panel), stripping and re-probing with α-SapA (lower panel).

5.4.4. Variability of surface layer proteins expression among *C. fetus* strains

As described in chapter 5.4.1. Western blot analysis of tyrosine-phosphorylation patterns of *Campylobacter* infected cells revealed phosphorylation of the *C. fetus* 97-3574 SapA protein. No bacterial phospho-proteins were detected after infection with *C. fetus* 5361 (Figs. 28A and 30A, B). To see if clinical isolates express SapA, the total bacterial-cell lysates from *C. fetus* 5361 (Magdeburg, Germany) and *C. fetus* 97-2126 (Young *et al.,* 2000) along with *C. fetus* 97-3574 strain (Young *et al.,* 2000) were subjected to SDS-PAGE/Coomassie staining and immunoblotting with α-SapA antibody. While the 97 kDa SapA protein was detected as one major band in *C. fetus* 97-2126 and *C. fetus* 97-3574 strains, other SapA protein species of 130 kDa and 112 kDa, were also observed as less prominent bands (Fig. 32A, B). To confirm that these proteins correspond to SapA protein species, the proteins were excised from Coomassie-stained gel and subjected to MALDI-MS which was performed by cooperation partner Dr. Sophie Haebel (Interdisciplinary Research Center for Mass Spectrometry of Biopolymers, University of Potsdam, Germany). Using this approach the 130 kDa and 112 kDa proteins were identified as *C. fetus* surface array protein SapA6 (accession no. Q841Y8), with 22% sequence coverage and *C. fetus* surface array protein SapA2 (accession no. Q53505), with 33% sequence coverage, respectively (data not shown). In agreement with the previous findings (Fig. 30B), *C. fetus* 5361 cell lysate failed to react with α-SapA antibody, suggesting that this strain lost its S-layer, during *in vitro* culturing, a phenomenon previously reported for other *C. fetus* strains (Fujita & Amako, 1994) (Fig. 32B).

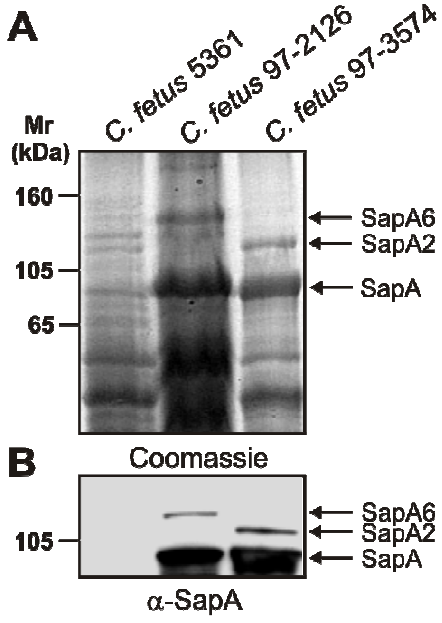
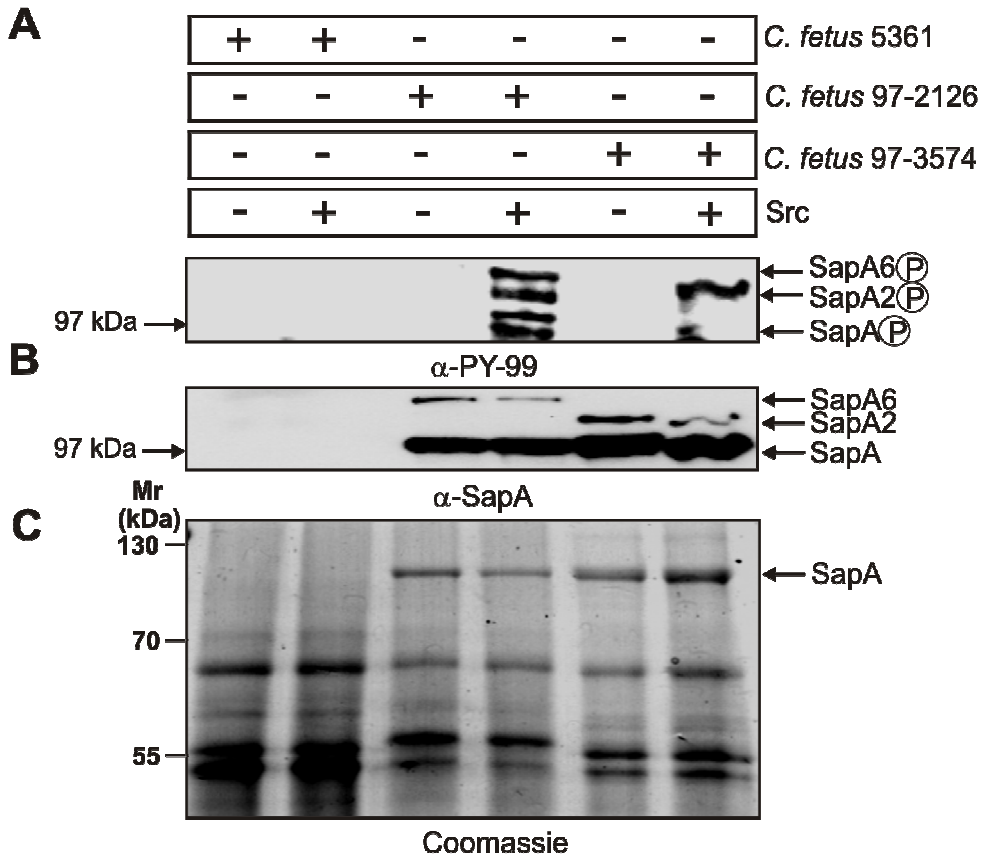


Fig. 32. Surface layer protein expression is variable among *C. fetus* strains. Cell lysates of indicated *C. fetus* isolates were analyzed by (A) SDS-PAGE/Coomassie staining and (B) immunoblotting with α-SapA antibody. Arrows show 97 kDa protein bands corresponding to the SapA proteins and additional bands which were identified as SapA6 and SapA2 proteins (see text). These bands were not detected for *C. fetus* 5361 strain.

To check whether SapA of different *C. fetus* isolates can be phosphorylated by recombinant c-Src kinase, *in vitro* kinase assays were performed. For this purpose cell lysates of *C. fetus* 5361, 97-2126 and 97-3574 strains were co-incubated with recombinant c-Src kinase followed by Western blot analysis with α-PY-99 and α-SapA antibodies. While SapA phosphorylation signal was detected with *C. fetus* 97-2126 and 97-3574 lysates no signal was observed with *C. fetus* 5361 (Fig. 33A). In addition, phosphorylation of SapA6 and SapA2 proteins was observed with *C. fetus* 97-2126 and 97-3574 lysates, respectively (Fig. 33A). As control, reaction without recombinant kinase was unable to phosphorylate SapA (Fig. 33A). This clearly indicates that c-Src kinase can phosphorylate SapA *in vitro* of both *C. fetus* 97-2126 and 97-3574 strains.



SapA from different *C. fetus* strains can be phosphorylated by c-Src *in vitro.* (A) Incubation of indicated *C. fetus* lysates with recombinant human c-Src in an *in vitro* kinase reaction resulted in specific phosphorylation of SapA, SapA2 and SapA6 proteins. SapA phosphorylation was neither detected in *C. fetus* 5361 lysate incubated with c-Src nor in lysates incubated without c-Src. (B) The expression of SapA proteins was verified by immunoblotting using the α-SapA antibody. (C) Coomassie staining presenting equivalent amounts of protein in each lane.

5.4.5. SapA-coated beads bind to INT-407 cells

Since the SapA protein is expressed on *C. fetus* surface, it may act as an adhesin/invasin during *C. fetus* infection. To test this hypothesis, latex beads were coated with SapA-GST, GST or BSA, used as negative controls, and their abilities to interact with INT-407 cells were investigated. SapA-GST-coated latex beads bound significantly more abundantly to INT-407 as compared to BSA-coated latex beads after 1-3 hrs of incubation (Fig. 34A-E, G-I, arrows). Whereas more SapA-GST-coated beads than GST-coated beads bound to INT-407 cells, the difference didn't reach statistical significance (Fig. 34A-F, I).

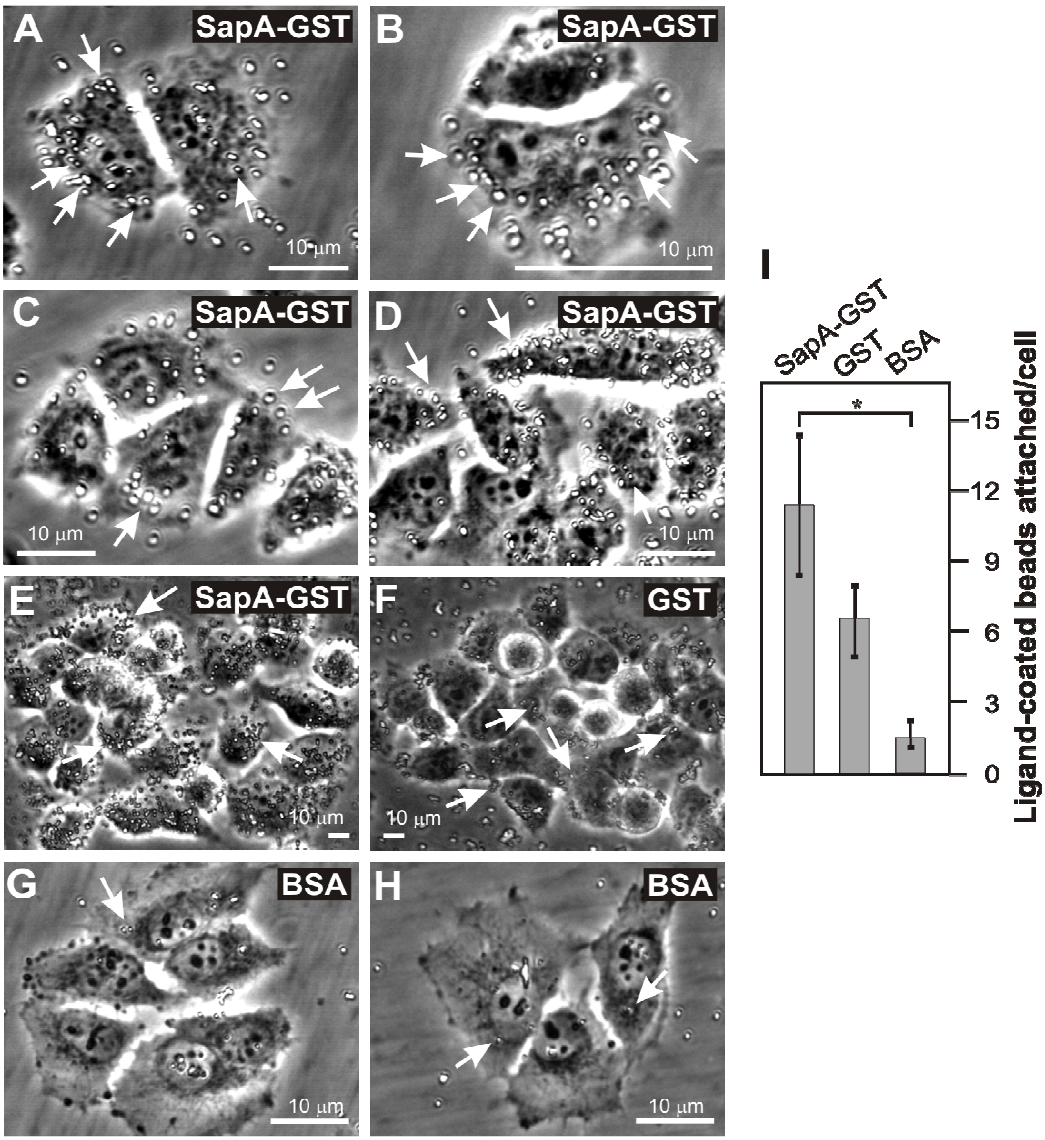


Fig. 34. Attachment of SapA-coated beads to INT-407 cells. Cell monolayers were co-incubated with (A-E) SapA-GST, (F) GST- (G-H) BSA-coated latex beads at an approximate cell:bead ratio of 1:200 for (A-D) 1 h or (E-H) 3 hrs. After gentle washing to remove unattached beads, cells were subjected to phase contrast microscopy. Arrows indicate examples of attached beads. (I) The number of adherent beads was counted in 25 randomly selected cells, based on micrographs obtained. (*) $P<0.05$ was considered as statistically significant.

5.4.6. INT-407 cells do not bind to SapA-coated surfaces

By analogy to known bacterial adhesins or invasins (Pizarro-Cerda & Cossart, 2006), it was assumed that SapA may induce host cell binding of *C. fetus*. To test this hypothesis cell attachment assays with SapA-GST were performed. In contrast to fibronectin (Fn)-coated wells, which were used as positive control (Fig. 35A, E), INT-407 cells did not bind to SapA-GST-coated plastic surface (96-well plates). No differences were observed between the level of adhesion of INT-407 cells to SapA and on the GST or BSA negative controls (Fig. 35B-E), indicating that SapA does not promote adhesion to host cells. Moreover, INT-407 cells were able to spread on Fn but not on SapA, GST or BSA (Fig. 35A-D).

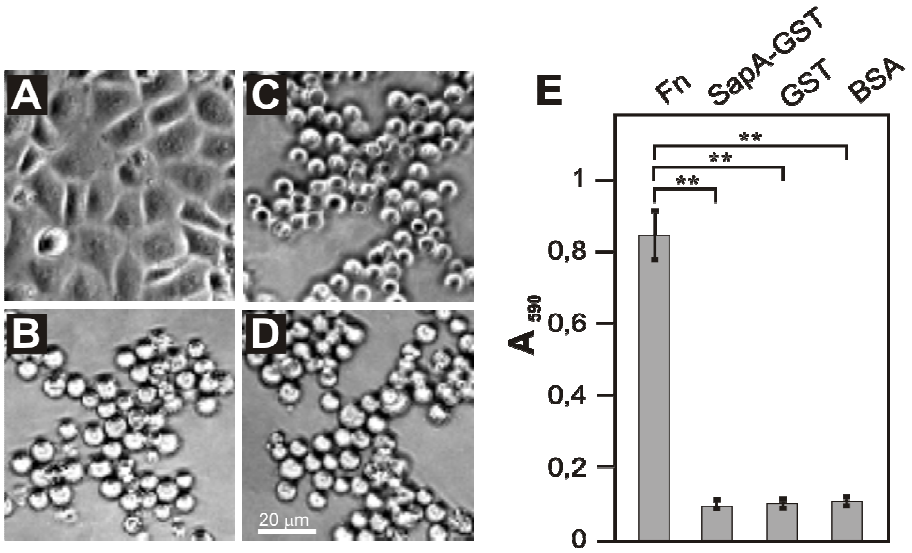


Fig. 35. INT-407 cells binding on different ligands. Wells were coated with 50 µg of (A) Fn (positive control), (B) SapA-GST, (C) GST and (D) BSA (negative controls). INT-407 cells were added and allowed to bind for 4 hrs. (A-D) Adherence of INT-407 cells was assessed by phase contrast microscopy. (E) The number of bound cells was quantified by crystal violet staining and subsequent absorbance measurement (Materials & Methods). (**) $P<0.005$ was considered as statistically significant.

5.4.7. SapA-coated beads are internalized into INT-407 cells

In the next experiment it was tested whether SapA-coated beads can be taken up by the INT-407 cells to investigate if SapA plays a role in invasion process. For this purpose INT-407 cells were incubated with SapA-GST- and GST-coated beads for 6 hrs and then they were subjected to differential IF-staining with α-SapA antibody. Whereas SapA-GST-coated beads attached to INT-407 cells and ECM with higher frequency as GST-coated control beads, the difference didn't reach statistical significance (Fig. 36A-G) confirming previous observations (Fig. 34I). Notably, SapA-GST coated beads were internalized more abundantly into INT-407 than GST-coated beads (Fig. 36A-G), indicating that SapA is important for host cells entry.

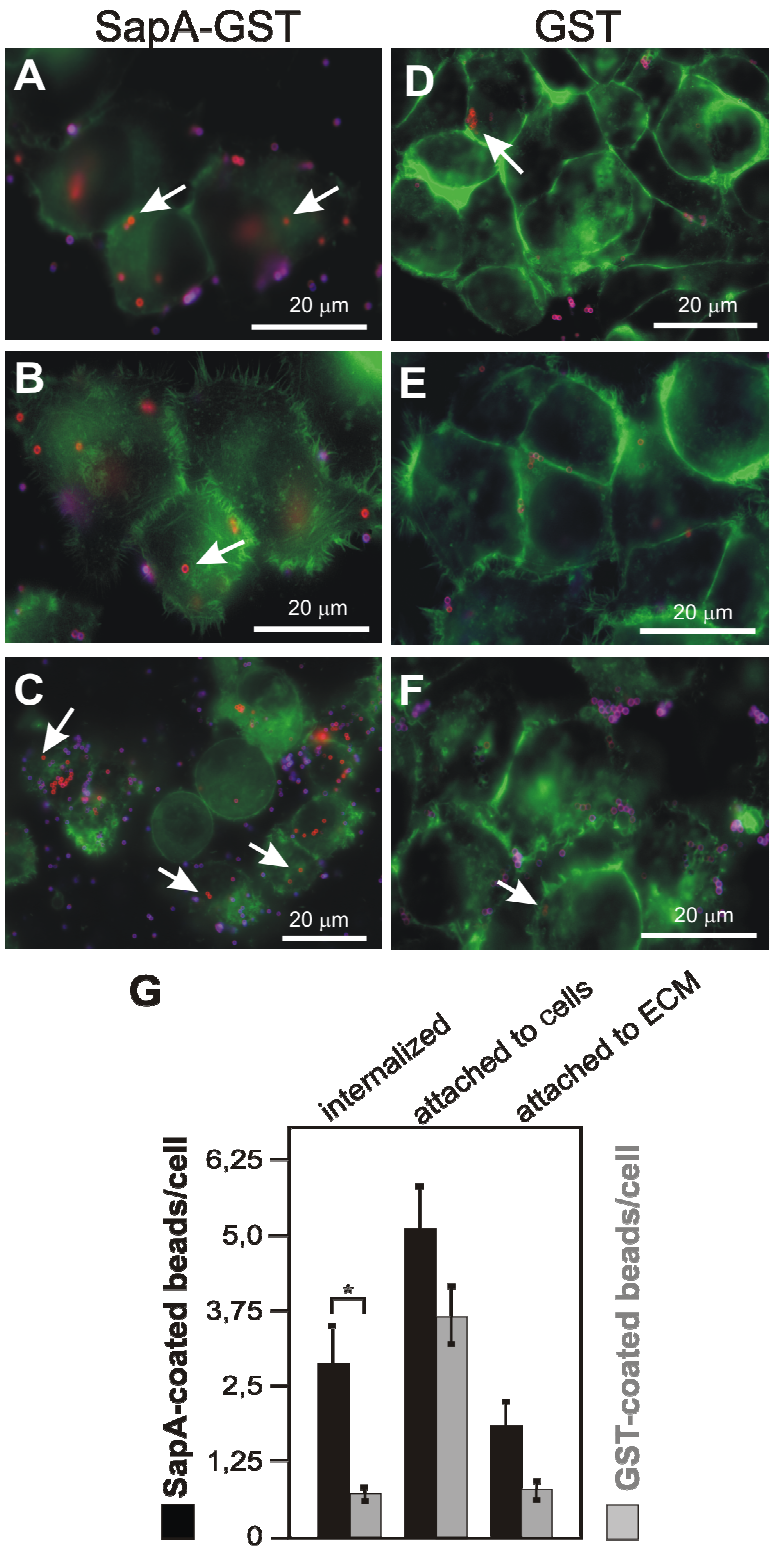


Fig. 36. Internalization of SapA-coated beads into INT-407 cells. Cell monolayers were co-incubated with (A-C) SapA-GST-, (D-F) GST-coated latex beads at an approximate cell:bead ratio of 1:100 for 6 hrs. After gentle washing to remove unattached beads, cells were subjected to differential IF staining and IF microscopy (Materials & Methods). Intracellular beads were probed with α-SapA antibody (red), extracellular beads were double stained with SapA-antibody (red and blue), actin was stained with FITC-phalloidin (green). Arrows indicate examples of internalized beads. (G) The number of internalized and attached beads was counted in 80 randomly selected cells, based on micrographs obtained. (*) $P<0.05$ was considered as statistically significant.

5.4.8. Importance of SapA expression during infection of host cells with *C. fetus*

To determine the contribution of the SapA protein in adhesion and invasion of *C. fetus* during infection of INT-407 cells, the interactions of *C. fetus* SapA-non-expressing strains, carrying 13.3-kb chromosomal deletion in SapA promoter region, S1-, S2-, S3- and their respective wild type SapA-expressing strains S1+, S2+, S3+ (Fujita & Amako, 1994; Fujita *et al.,* 1997) were examined.

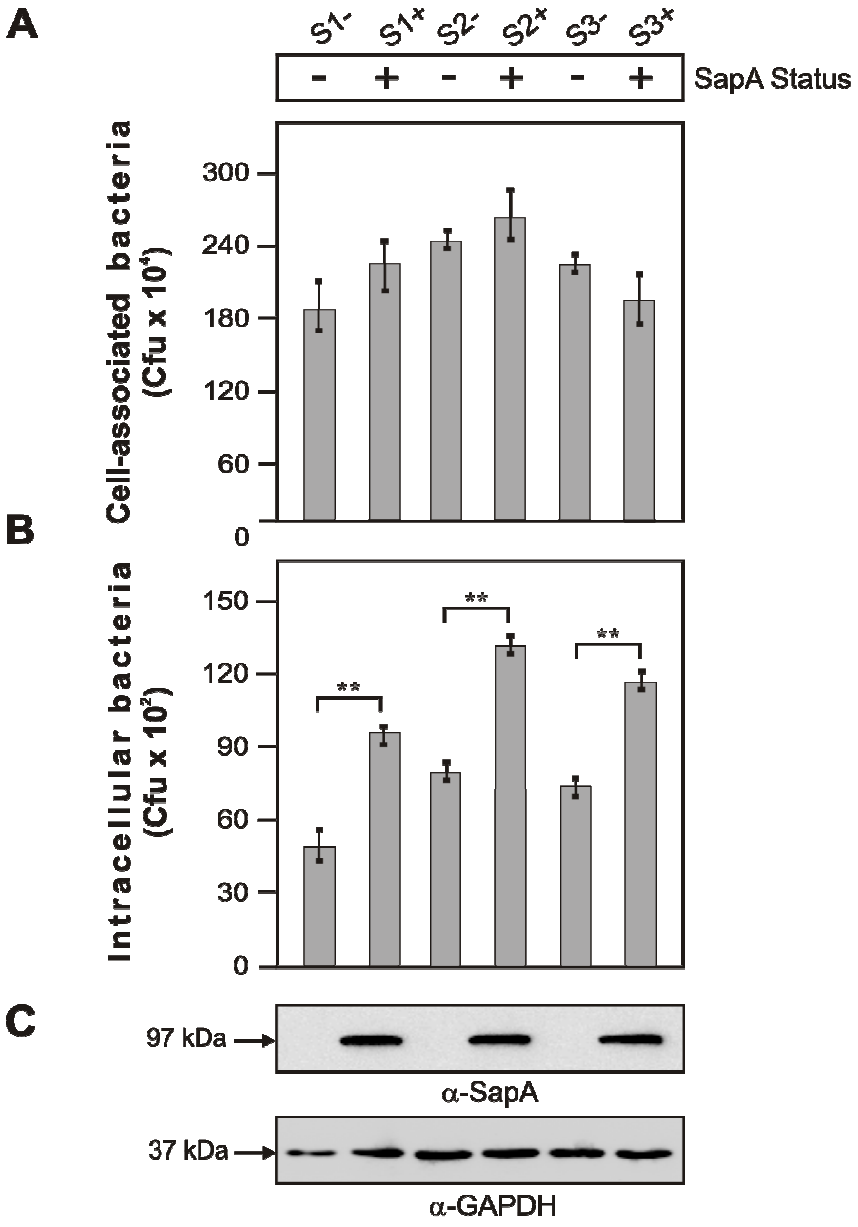


Fig. 37. Effect of SapA deficiency on cellular adhesion and invasion of *C. fetus*. INT-407 cells were infected for 6 hrs with indicated *C. fetus* SapA-expressing and SapA-non-expressing strains. (A) Total cell associated and (B) intracellular *Campylobacter* cells were quantified by gentamicin protection assays. (**) $P \leq 0.001$ were considered as statistically significant. (C) The expression of SapA protein was verified by immunoblotting using the α-SapA antibody. GAPDH expression levels were determined as control.

Whereas quantification of intracellular bacteria by the gentamicin protection assay revealed that the SapA-deficient strains exhibited significantly lower invasion rates than their wild-type strains (Fig. 37B), no difference in binding of these strains was observed (Fig. 37A). Immunoblot analysis with α-SapA proved that the SapA protein was not expressed by either SapA-negative strains (Fig. 37C). These findings confirmed previous beads experiments results as described in chapters 5.4.5.-5.4.7, demonstrating that SapA is not essential for bacterial binding to host cells, but SapA appears to play a role in *C. fetus* host cell entry. The data clearly show that SapA is an important *C. fetus* pathogenicity factor.

5.4.9. Importance of SapA phosphorylation during infection of host cells with *C. fetus*

To further investigate the role of SapA phosphorylation by Src kinase during *C. fetus* infection, the pharmacological inhibitor PP2 was applied. While treatment of cells with PP2 significantly reduced invasion of *C. fetus* S1+, S2+ and S3+ strains, it had no effect on low invasion rates of *C. fetus* S1-, S2- and S3-, as quantified by gentamycin protection assay (Fig. 38, grey and black bars). Thus, Src and probably SapA phosphorylation by this kinase plays an important role in invasion of *C. fetus*.

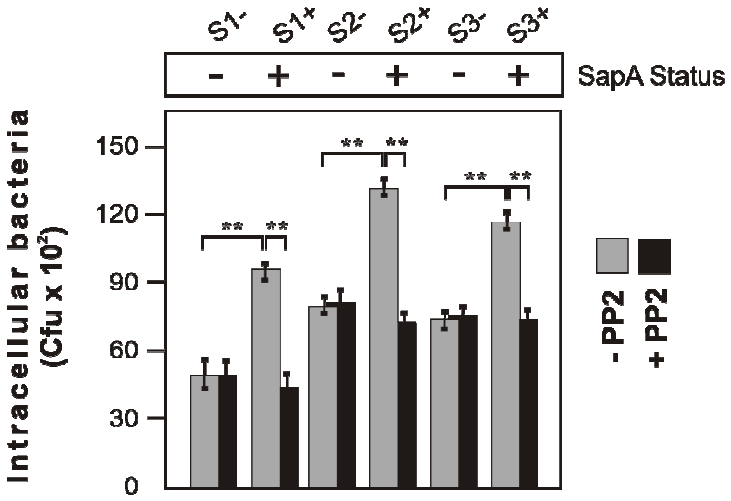


Fig. 38. Effect of lack of SapA phosphorylation by Src kinase on *C. fetus* invasion. INT-407 cells were infected for 6 hrs with indicated *C. fetus* SapA-expressing and SapA-non-expressing strains, in absence (grey bars) or presence (black bars) of PP2 inhibitor. Intracellular *Campylobacter* cells were quantified by gentamicin protection assays. (**) $P \leq 0.001$ were considered as statistically significant.

5.4.10. SapA triggers IL-8 secretion

To investigate whether recombinant SapA is a biologically active protein which may influence immunoregulatory signaling, its ability to induce IL-8 release was tested by standard ELISA. Significant difference in IL-8 secretion was observed between cells co-incubated with SapA-

GST- and GST-coated beads (Fig. 39), indicating that SapA exhibits the capability to trigger IL-8 secretion of host cells.

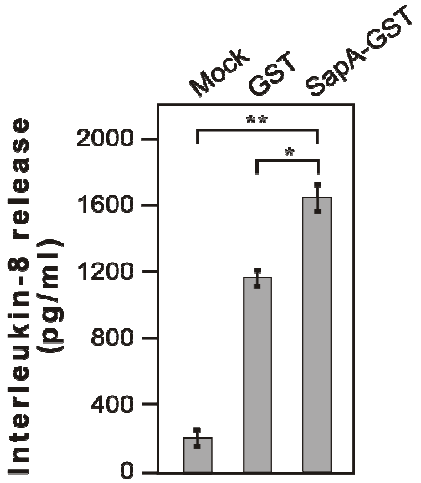


Fig. 39. Purified recombinant SapA is able to induce IL-8 secretion of host cells. Mouse fibroblast monolayers were co-incubated with SapA-GST- and GST-coated latex beads at an approximate cell:bead ratio of 1:100 for 6 hrs. IL-8 release into the culture supernatant was determined by standard ELISA. (*) $P \leq 0.05$ and (**) $P \leq 0.005$ were considered as statistically significant.

Next, the role of SapA protein expression for the induction of IL-8 release during *C. fetus* infection was examined. For this purpose, INT-407 cells were infected with *C. fetus* SapA-expressing strains and respective SapA-deficient strains, and their capability to trigger IL-8 secretion was compared. *C. fetus* SapA-non-expressing strains (S2- and S3-) showed reduced ability to induce IL-8 release from INT-407 in comparison to wt *C. fetus* S2+ and S3+, respectively (Fig. 40). These statistically significant ($P \leq 0.001$) data further confirm that SapA is important pathogenicity factor triggering IL-8 secretion from host cells during *C. fetus* infection.

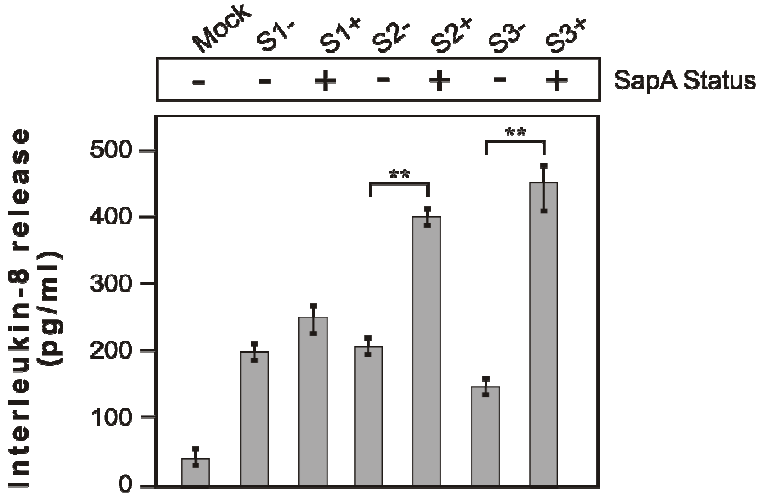


Fig. 40. Effect of SapA on induction of IL-8 secretion from infected host cells. INT-407 cells were infected for 24 hrs with indicated *C. fetus* SapA-expressing and SapA-non-expressing strains. IL-8 release into the culture supernatant after infection was determined by standard ELISA. (**) $P \leq 0.001$ were considered as statistically significant.

5.4.11. *Campylobacter fetus* binding and invasion occurs on the edges of and between INT-407 cells, as revealed by FESEM

The interaction of *C. fetus* S2+ and S2- strains with the surface of INT-407 epithelial cells was analyzed by FESEM, performed by Dr. M. Rohde. One hundred infected cells were investigated in each experiment. Analysis revealed that the bacteria were able to bind and invade into the host cells (Fig. 41A-F, red arrows). Interestingly, it was found that the bacteria attached to the cells in a very specific manner. Whereas *C. fetus* S2+ was regularly observed in association with the edges of and between the infected cells, the *C. fetus* S2- was only rarely seen bound to cells.

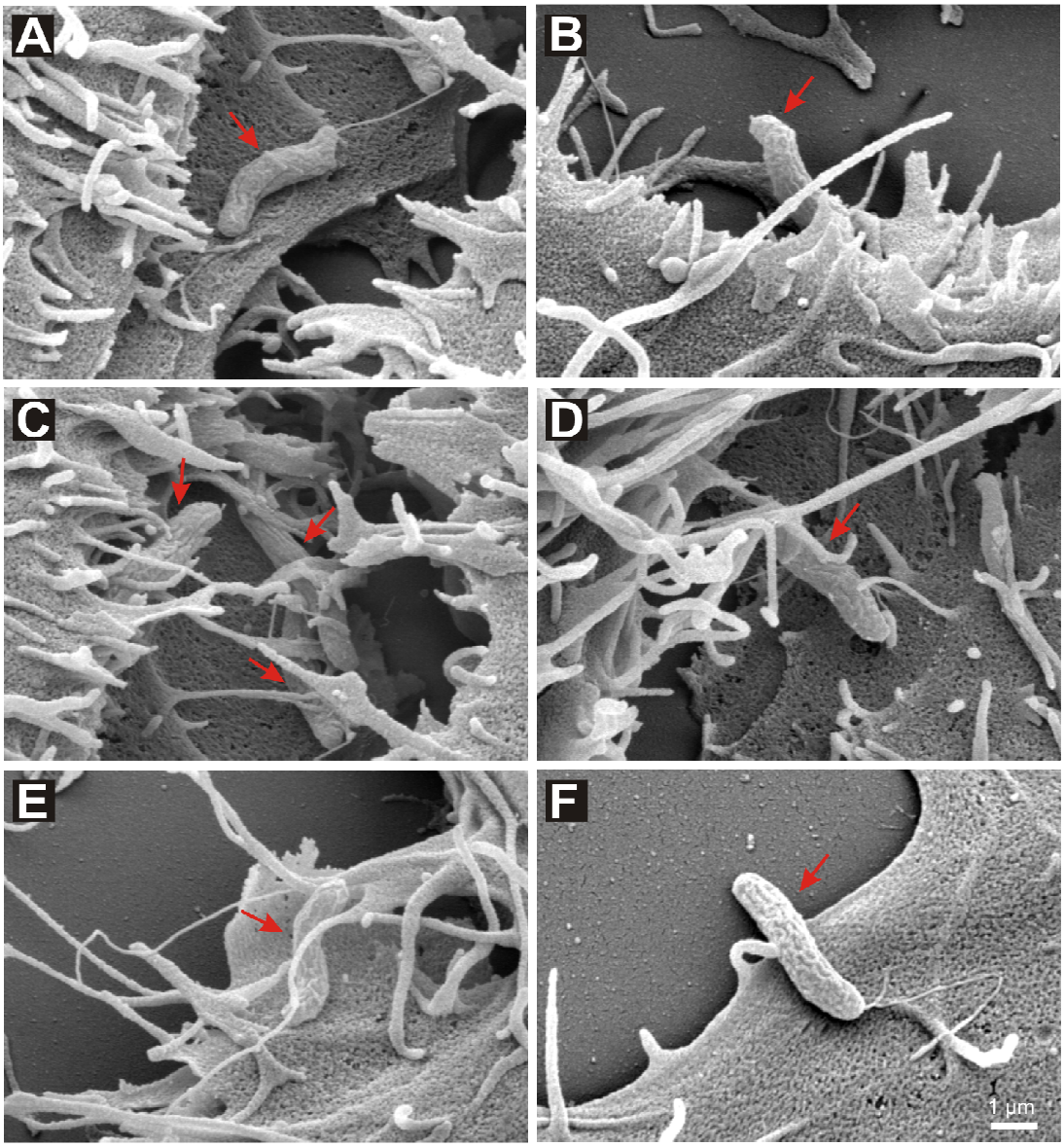


Fig. 41. High resolution field emission scanning electron microscopy of *C. fetus* adhesion and invasion into INT-407 epithelial cells after 4 hrs of infection. (A-E) *C. fetus* S2+ was often found on the edges of and between the cells. (F) *C. fetus* S2- was rarely observed bound to cells. Arrows indicate examples of *C. fetus*.

6. Discussion

6.1. Role of CadF protein in *Campylobacter jejuni* and *Campylobacter coli*

The ability of pathogenic bacteria to bind to host tissues is important as it represents an early event in the establishment of an *in vivo* niche. In some instances, such binding is also a prerequisite for host cell invasion, where the organisms are protected intracellularly from the humoral and cellular immune responses of the host (Alrutz & Isberg, 1998; Cossart & Sansonetti, 2004). The common theme among pathogenic micro-organisms is their ability to utilize host cell molecules during the infectious process to facilitate their binding and entry into host cells (Watarai *et al*., 1996, Pizarro-Cerda & Cossart, 2006). The pathogenicity of several *Campylobacter* species is dependent on their ability to attach and invade the human intestine (Kopecko *et al.,* 2001; Mooney *et al.,* 2003). One of the adhesion factors that *C. jejuni* uses to attach, and eventually invade mammalian cells, is CadF, a protein that binds to fibronectin (Fn) – a component of the extracellular matrix (Konkel *et al.,* 1997). *In vitro* assays revealed that the binding of *C. jejuni* clinical isolates to immobilized Fn can be reduced using a α-CadF polyclonal serum (Monteville *et al*., 2003). Most recently, a single surface-exposed domain was identified within the CadF protein that binds to the Fn. Using an overlapping peptide library derived from CadF, maximal Fn-binding activity was localized within four amino acids (aa 134-137) consisting of the phenylalanine-arginine-leucine-serine motif (Konkel *et al.,* 2005). Furthermore, the importance of CadF for the adherence of *C. jejuni* to epithelial cells has been demonstrated *in vitro* (Konkel *et al.,* 1997). Finally, the biological significance of the CadF as an adhesin has been validated upon performing *in vivo* studies. Ziprin and co-workers (Ziprin *et al*., 1999; 2001) reported that *C. jejuni cadF* mutant is unable to colonize the intestinal tract of Leghorn chickens, thus providing evidence that this 37 kDa outer-membrane protein plays an *in vivo* role in mediating the organism's binding to the intestinal epithelium.

One of the aims of my study was to determine the genetic and functional diversity of CadF protein among a large number of *C. jejuni* and *C. coli* isolates. Therefore, protein separation, blotting and staining with two highly specific α-CadF antibodies was performed. The expected, prominent 37 kDa CadF protein (p37) was detected in all tested *C. jejuni* and *C. coli* isolates. In addition, Western blot analysis indicated second protein species of 32 kDa (p32) that was mostly less prominent. The results, which are consistent and extend earlier observations (Konkel *et al.,* 1997; 1999a), also revealed both that the number and intensity of CadF bands varied among *C. jejuni* strains. While p37 was detected in all *C. jejuni* isolates of human and animal origin, the less prominent p32 band was found only in 62% of the *C. jejuni* isolates of human origin and in 96% of the *C. jejuni* of animal origin.

To prove the specificity of the α-CadF antibodies, CadF expression pattern of two isogenic *cadF* knockout mutants was tested. By lack of staining it was shown that p32 and p37 are not expressed in the *cadF* mutants. This confirms that both bands indeed correspond to CadF translation products. Heat-modifiability is a well-known feature of outer membrane proteins (Nakamura & Mizushima, 1976; Bolla *et al.,* 1995), including CadF (Konkel *et al.,* 1997; 1999a; Mamelli *et al.,* 2006; 2007). The differences in conformational states of membrane proteins are readily apparent by SDS-PAGE, where the native protein exhibits a faster migration than the denaturated form of the protein. Further boiling in SDS-containing sample solubilization buffer results in a complete disruption of protein folding and a change in the apparent molecular weight (Nakamura & Mizushima, 1976; Sugawara *et al.,* 1996). Therefore, the migration of CadF as two protein species is likely caused by their heat-modifiable conformational state, where p32 is the incompletely denaturated and partially folded form of CadF.

In contrast to earlier reports, where the CadF protein was found to be conserved in size and antigenicity among *C. jejuni* and *C. coli* isolates from USA (Konkel *et al.,* 1999a), it was observed that all *C. coli* isolates tested in my study possessed a larger CadF (p39 and p34) than *C. jejuni*. Sequence analysis of three *C. coli* isolates confirmed this difference between species and indicated that *C. coli* carried a stretch of 13 amino acids in the middle region of the protein. Interestingly, the latter insertion sequence was not found in one *C. coli* isolate from the USA which instead contained another insertion sequence of 7 amino acids (Konkel *et al.,* 1999a). However, whether the differences in amino acid sequence or a lower expression level accounted for the apparent weaker immunoreactivity of the *C. coli* CadF with the polyclonal antisera remains to be determined. Nevertheless, the data strongly suggest that the differences in molecular size and differences in nucleotide sequence between the *C. jejuni* and *C. coli* isolates may be a suitable diagnostic marker to discriminate between these species in food and clinical specimen.

The possible biological significance of the variation in CadF was investigated by comparing a subset of *C. jejuni* and *C. coli* strains for their ability to infect INT-407 intestinal epithelial cells, which serves as an *in vitro* model system for *C. jejuni* and *C. coli* attachment and invasion (Hu & Kopecko, 1999; Biswas *et al.,* 2000; Monteville *et al.,* 2003; Nadeau *et al.,* 2003; Konkel *et al.,* 2004; Hu *et al.,* 2005). Interestingly, *C. jejuni* strains adhered and invaded INT-407 cells at significantly greater levels than *C. coli* strains. This effect was at least in part caused by CadF as the 81116Δ*cadF* and F38011Δ*cadF* mutants showed reduced adhesion, which is consistent with previous studies showing a reduced adherence to INT-407 cells of a *C. jejuni cad*F mutant (Konkel *et al.,* 1997; Monteville *et al.,* 2003). These results may indicate that *C. coli* CadF is less functional than its *C. jejuni* counterpart, although one cannot exclude that additional differences between the subspecies could be

involved (Wassenaar & Blaser, 1999). Furthermore, a significant reduction in invasion of INT-407 cells was noted with both *C. jejuni cadF* mutants when compared with wild-type isolates, confirming that CadF is a major pathogenicity factor of *Campylobacter*, which is not only important for bacterial binding but also required for maximal invasion of intestinal epithelial cells.

6.2. Rac1 and Cdc42 as central players mediating *Campylobacter jejuni* invasion

Host cell invasion is an essential process in the pathogenesis of many bacteria, including *C. jejuni* (Kopecko *et al.,* 2001). The extracellular milieu can be a harsh environment in which pathogens are subjected, in addition to physical stresses (such as low pH or shear stress imposed by flow of mucosal secretions or blood), to many other host defense mechanisms including cellular exfoliation, complement deposition, antibody labeling and subsequent recognition by macrophages or cytotoxic T cells (Pizarro-Cerda & Cossart, 2006). Several bacterial species have evolved molecular strategies to actively induce their entry into target cells for replication and/or dissemination to other host tissues. For example, organisms, such as the enteropathogenic *Yersinia* and *Salmonella* appear to utilize cellular entry to gain access to subepithelial regions. Whereas *Neisseria meningitis* may use specific uptake factors to directly access deep tissue sites, *Shigella* or *Chlamydia* must replicate within host cells to cause disease (Sansonetti *et al.,* 1999). Once microorganisms translocate to epithelium, the routes that different organisms take to promote disease may diverge significantly from one to another. Invasion involves numerous steps: bacterial binding at specific receptor sites, signaling to the host cell, modification of intracellular host signal transduction pathways, membrane and cytoskeletal rearrangements, and eventual engulfment of the bacterium, which commonly involves the activity of one or more of the host cell proteins of small Rho family GTPases (Gruenheid & Finlay, 2003; Cossart & Sansonetti, 2004; Rottner *et al.,* 2004). Rho family members are small GTP-binding proteins that induce a variety of host cell responses (Nobes & Hall, 1995; Caron & Hall, 1998; Tran Van Nhieu *et al.,* 1999; Cossart & Sansonetti, 2004). They are known principally for their pivotal role in regulating the actin cytoskeleton, but their ability to influence cell polarity, microtubule dynamics, membrane transport pathways and transcription factor activity is probably just as significant (Ettiene-Manneville & Hall, 2002). They cycle between a GDP-bound (inactive) state and GTP-bound (active) state, and, in the GTP-bound state, the GTPase relays extracellular signals to a large number of downstream effectors. RhoA, Rac1 and Cdc42, the best-characterized members of the family, have distinct effects on the actin cytoskeleton (Ridley *et al.,* 1992; Olson *et al.,* 1995; Caron & Hall, 1998). It has been shown that Rho

proteins are involved in formation of stress fibers and focal adhesion complexes, whereas Rac1 triggers lamellipodia and membrane ruffling, and Cdc42 proteins induce filopodia formation (Nobes & Hall, 1995). It is important to note that the role of Cdc42 is still not fully clear because Cdc42-deficient fibroblast cells are still capable to induce the formation of filopodia and lamellipodia (Czuchra *et al*., 2005). During cell spreading, Rho family members function sequentially, with initial activation of Cdc42, followed by Rac1 and RhoA (Ridley *et al.,* 1992; Nobes & Hall, 1995). However, in other actin-dependent processes such as bacterial invasion, a distinct subset of Rho GTPases becomes activated, often in a cell-type specific manner. For example, it has been demonstrated that Rac1 and Cdc42 play a crucial role in the invasion of *Salmonella enterica* and *Shigella flexneri* (Hardt *et al.,* 1998; Tran Van Nhieu *et al.,* 1999), whereas RhoA is important for the uptake of *Mycobacterium avium* (Sangari *et al.,* 2000) or *Pseudomonas aeruginosa* (Kazmierczak *et al.,* 2001). Rac1 is required for internalization of *Chlamydia trachomatis* (Carabeo *et al.,* 2004), *Listeria monocytogenes* (Seveau *et al.,* 2007) and *Yersinia pseudotuberculosis* (Alrutz *et al.,* 2001). Invasion of enteropathogenic *Escherichia coli* (EPEC) has been shown to be Cdc42 dependent (Kenny *et al.,* 2002; Jepson *et al.,* 2003). However, the role of Rho GTPases in *C. jejuni* invasion is not known.

The growing number of known bacterial virulence factors acting on small Rho family GTPases comprises GEFs and GAPs, which are capable of inducing localized signaling to actin rearrangement at the sites of bacterial invasion (Gruenheid & Finlay, 2003; Cossart & Sansonetti, 2004; Rottner *et al.,* 2004), or a novel class of type III effector proteins which mimic Rho GTPases (Alto *et al*., 2006). In addition, numerous bacterial protein toxins are specialized to activate, inhibit or modify Rho family GTPases (Barbieri *et al*., 2002; Barbieri & Aktories, 2005). Thus, conceptually, the latter toxins can modulate many aspects of actin-cytoskeletal function and provide a powerful tool to study the role of GTPases during the invasion of other pathogens, including *C. jejuni.*

Here, inhibition of endogenous Rho-family members by compactin and *Clostridium difficile* toxin B and TcdB (Just *et al*., 1995) effectively reduced the internalization of *C. jejuni*, suggesting an involvement of these proteins in the invasion process. Inactivation of Rac and R-Ras but not Rho and Cdc42 by TcdBF (Chaves-Olarte *et al.,* 2003) also showed a blocking effect indicating that Rac but not Rho is involved in the entry process of *C. jejuni.* Treatment of host cells with exoenzyme C3 from *Clostridium botulinum*, which specifically inactivates RhoA-C (Aktories, 1997; Genth *et al.,* 2003; Barbieri & Aktories, 2005) did not inhibit *C. jejuni* uptake, confirming that RhoA-C does not play a role in this process. Furthermore, both the transient transfection of DN-Rac1 or DN-Cdc42 and down regulation of Rac1 and Cdc42 but not RhoA using target-specific siRNA led to significant reduction of *C. jejuni* invasion. In turn, internalization of *C. jejuni* was enhanced significantly through activation of endogenous Rac1

and Cdc42 but not RhoA. Whereas treatment of host cells with the *E. coli* CNF-1, which has the ability to permanently activate all three GTPases by deamidation of glutamine residues (Flatau *et al*., 1997; Schmidt *et al*., 1997; Lerm *et al*., 1999), promoted *C. jejuni* uptake, exposure to CNF-Y, a specific activator of RhoA, did not lead to increased invasion. Additionally, transient expression of CA-Rac1 and/or CA-Cdc42 confirmed previous results. It is important to note that in comparison to CA-Cdc42, CA-Rac1 transfection had a much more pronounced stimulatory effect on *C. jejuni* uptake. These finding could suggest that Rac1 may be the major player regulating host cell internalization of *C. jejuni*.

Interestingly, expression of CA-RhoA reduced the levels of *C. jejuni* invasion. Numerous studies have shown that CA-RhoA or endogenous RhoA activation inhibited Rac1 activation in neurons (Yamaguchi *et al.,* 2001; Negishi & Katoh, 2002) and CHO cells (Sugimoto *et al.,* 2003). Activation of Rho kinase by RhoA induces the phosphorylation and activation of myosin, located at the cell periphery, and consequently elevates contractile activity of actomyosin, enhancing the stabilization of cortical actin filaments. This stabilization disturbs the Rac1-mediated reorganization of the actin cytoskeleton at the cell periphery and subsequent protrusion (Negishi & Katoh, 2002). Thus, activated RhoA may block Rac1 resulting in blocking of *C. jejuni* host cell entry. However, if the latter signaling occurs in INT-407 epithelial cells, remains to be identified. In summary, the data described above indicate that Rac1 and Cdc42 but not RhoA are involved in *C. jejuni* invasion. This idea was further confirmed by the finding that Rac1 and Cdc42 are prominently activated in INT-407 cells between 2-4 hrs after infection with *C. jejuni*. This is also consistent with the observation that high rates of *C. jejuni*-induced membrane ruffling and invasion can be detected as soon as 4-6 hrs after infection.

Changes of actin-dependent membrane dynamics generated by active Cdc42 and Rac1 have been shown to be associated with the uptake of numerous other invasive pathogens into non-phagocytic mammalian cells, including *S. flexneri* (Tran Van Nhieu *et al.,* 1999) and *S. enterica* (Hardt *et al.,* 1998). Invasion is thereby accompanied by extensive membrane ruffling and requires different Rho GTPases including Rac1 and Cdc42, which are regulated directly through bacterial factors injected into the host cell by a T3SS (“trigger mechanism”) (Hardt *et al.,* 1998; Tran Van Nhieu *et al.,* 1999; Cossart & Sansonetti, 2004). However, other than the flagella, *C. jejuni* does not encode a classical T3SS (Konkel *et al*., 1999; Hofreuter *et al*., 2006). In contrast, the “zipper mechanism” of invasion used by *Listeria* and *Yersinia* involves direct contact between the bacterium and the host cell surface and results in a tight association between bacterial ligands and host receptors. For engulfment of the bacterium, localized actin-cytoskeletal reorganization is also required (Finlay & Cossart, 1997; Lecuit *et al*., 1997). As the cell surface rearrangements observed during *C. jejuni* invasion were not so pronounced as in case of *S. flexneri* (Adam *et al.,* 1995; 1996; Tran Van

Nhieu *et al.,* 1999) or *S. enterica* (Hardt *et al.,* 1998; Zhou *et al.,* 1999; 2001), my electron microscopic studies support the view that *C. jejuni* did not enter host cells by a classical trigger-like mechanism.

6.3. Involvement of CadF in *Campylobacter jejuni*-induced activation of Rac1 and Cdc42

Whereas the entry of *Yersinia* and *Listeria* can be mediated by a single bacterial protein, the invasins InvA (Isberg & Tran Van Nhieu, 1994) and INlA or INlB (Lecuit *et al.,* 1997; Braun *et al.,* 1998), respectively, invasion of *Salmonella* and *Shigella* is a multifactorial process with more than 20 bacterial proteins being involved (Finlay & Falkow, 1997). In case of *C. jejuni*, it was tempting to propose that known pathogenicity factors such as plasmid pVir, CDT, KpsS, WaaF, PEB1 or CadF may be involved in *C. jejuni*-induced GTPase activation. Thus, isogenic mutants of the respective genes or strains lacking pVir were used in order to investigate the role of one or the other bacterial factor in this process.

A number of bacterial enteric pathogens contain plasmids that contribute to pathogenesis, including *Shigella* (Sasakawa *et al.,* 1992; Sansonetti & Egile, 1998), *Salmonella* (Chikami *et al.,* 1985; Guiney *et al.,* 1995), and EPEC (Tobe *et al.,* 1999). No evidence was seen for the involvement of plasmids in the virulence of *C. jejuni* until Bacon and co-workers (Bacon *et al.,* 2000) identified pVir in strain 81-176. pVir is a plasmid encoding homologues of components of T4SS (Bacon *et al*., 2000; 2002) known to be important for the virulence of a number of major bacterial pathogens (Backert & Meyer, 2006). pVir plasmid was shown to be involved in the virulence of *C. jejuni* both *in vitro* and *in vivo* (Bacon *et al.,* 2000). Recently, pVir was identified in 17 of 104 (17%) clinical isolates and associated with occurrence of blood in patients stool and more severe invasive *Campylobacter* infections (Tracz *et al.,* 2005). In contrast to this communication, Louwen and co-workers (Louwen *et al.,* 2006) reported the absence of an association with the plasmid pVir in patients infected with *C. jejuni* who developed bloody diarrhea. Interestingly, wt *C. jejuni* strains, 84-25 and F38011 tested in this study, which lack pVir, induced the activation of Rho GTPases, suggesting that pVir and the encoded factors ComB3 and VirB11 are not involved in the invasion of *C. jejuni* 81-176 into host cells. Furthermore, certain genes such as *kpsS* and *waaF* which play a role in the biosynthesis of CPS and LOS, respectively, have been shown previously to be implicated in *C. jejuni* invasion (Karlyshev *et al.,* 2000; Bacon *et al.,* 2001; Kanipes *et al.,* 2004). Here it was found that their respective isogenic mutants activated Rho GTPases comparable to wt *C. jejuni*, suggesting that *kpsS* and *waaF* genes may have other functions during the invasion process. The cytolethal distending toxin (CDT) is also considered as an important *C. jejuni* virulence factor (Hickey *et al.,* 2000).

The *cdt*B gene encodes the active subunit of CDT, exerting its effect as a nuclease that damages DNA (Lara-Tajero & Galan, 2000). The *cdt*B isogenic mutant was able to activate Rho GTPases, indicating no role of CDT in Rho GTPases activation. In addition, inactivation of the important bacterial adhesin PEB1, previously reported to affect *C. jejuni* adherence to epithelial cells, as well as colonization of mice (Pei *at al.,* 1998), even enhanced *C. jejuni*-induced Rac1 and Cdc42 activation, suggesting that PEB1-dependent bacterial binding to host cells *per se* does not trigger GTPase activation. Importantly, I reproducibly found that an isogenic *cadF* mutant less efficiently induced the activation of Rho GTPases as compared to wt bacteria. This suggests that CadF, at least in part, is involved in Rac1/Cdc42 activation. Because CadF is a Fn-binding protein, it could be a bi-functional protein, acting not only as a canonical adhesin for bacterial binding to Fn but also stimulating integrins and growth factor receptor clustering, which subsequently could activate downstream factors such as GEFs of Rac1 and Cdc42.

6.4. Role of the ß$_1$ integrins in host cell invasion of *Campylobacter jejuni*

Integrins are large heterodimeric transmembrane proteins of multicellular organisms involved in a wide variety of adhesive functions such as cell-cell interaction, cell migration, differentiation and adhesion. Members of this receptor family are able to bind extracellular matrix proteins as well as cytoskeletal components, thus providing a sophisticated communication system between the extracellular environment and intracellular cytoskeleton (Hynes, 2002). Integrins are composed of two subunits, α and ß, that can heterodimerize to form more than 20 different receptors exhibiting specific patterns of expression (Schlaepfer & Hunter, 1998; van der Flier & Sonnenberg, 2001). Many mammalian cells express integrin heterodimers with a ß1 chain. In epithelial layers, members of this family are usually involved in binding to extracellular matrix components such as Fn, collagen and laminin (Isberg & Tran Van Nhieu, 1994). Several pathogens exploit their interaction with integrins not only to adhere to host cells but also to trigger actin-cytoskeletal rearrangements that can result in cellular invasion. For example, the invasin InvA, a surface protein of *Y. enterocolitica* and *Y. pseudotuberculosis* promotes cell attachment and entry by direct binding to at least five different members of the ß1 integrin receptor family (Isberg & Leong, 1990; Leong *et al.,* 1990). A number of other microbial pathogens are known to produce adhesive factors that bind ECM proteins and promote cell uptake by bridging ECM molecules. For instance, attachment and cell entry of *Neisseria gonorrhoeae*, *Streptococcus pyogenes*, and *Staphylococcus aureus* occur by interaction of their adhesins, *e.g.* Opa (Opacity-associated outer membrane proteins), Cpa (Collagen-binding protein of group A streptococci), EbpS (Elastin-binding protein of *Staphylococcus aureus*) with

vitronectin, collagen or elastin (Dehio *et al.,* 1998; Park *et al.,* 1999; Kreikemeyer *et al.,* 2005). Numerous bacteria have evolved surface proteins that possess the ability to specifically bind to Fn and exploit its interaction with integrin $\alpha_5ß_1$, the principal Fn receptor, including *M. avium* (Schorey *et al*., 1996), *N. gonorrhoeae* (van Putten *et al*., 1998), *S. aureus* (Jonsson *et al.,* 1991; Joh *et al.,* 1999; Agerer *et al*., 2003) and *S. pyogenes* (Ozeri *et al.,* 1998; 2001). In *C. jejuni*, the CadF protein mediates the binding of *C. jejuni* to Fn, however, the precise mechanism of bacterial invasion and the roles of Fn and integrins in this process have been not fully elucidated (Konkel *et al*., 1997; 2005). It is not clear whether Fn-coated *C. jejuni* engage integrins to promote its entry into host cells and how the binding of integrins is translated into an uptake signal. In my study, *C. jejuni* invasion of $ß_1$-deficient cells GD25 was found to be significantly reduced as compared to $ß_1$A expressing cells, demonstrating the essentiality of $ß_1$ integrin for uptake of this pathogen. *C. jejuni* is most probably able to invade eukaryotic cells by indirectly engaging ß1 integrin-containing host receptors, where Fn can act as a bridge and indirectly link the bacteria expressing CadF protein to integrins on human cells.

6.5. Importance of the focal adhesion kinase and CadF for Rac1 and Cdc42 activation and host cell invasion of *Campylobacter jejuni*

The short cytoplasmic tail of integrins possesses no enzymatic activity, hence integrins transduce signals by associating with adapter proteins that connect the integrin to the cytoskeleton, cytoplasmic kinases, and transmembrane growth factor receptors (Schwartz, 2001; Hynes, 2002). FAK is a non-receptor protein-tyrosine kinase (PTK) that indirectly localizes to sites of integrin-receptor clustering through carboxy-terminal-domain-mediated interactions (Hildebrand *et al.,* 1993) with integrin-associated proteins such as paxillin (Tachibana *et al.,* 1995; Liu *et al.,* 1999) and talin (Chen *et al.,* 1995). FAK becomes phosphorylated at seven to eight different tyrosine residues *in vivo* after engagement of integrin with matrix proteins (Schlaepfer & Hunter, 1996; Schlaepfer *et al.,* 2004). The amino-terminal domain of FAK contains an autophosphorylation site (Y397), which serves as a high-affinity binding site for the SH2 domain of Src family of PTKs *in vivo* (Schlaepfer *et al.,* 2004). Active FAK and Src PTKs phosphorylate paxillin, α-actinin and $p130^{CAS}$, a docking protein that recruits the adapter proteins Crk and Nck (Richardson & Parsons, 1996; Schlaepfer *et al.,* 1997). Phosphorylation of tyrosine residues in the FAK carboxy-terminal domain by Src kinases at Y925 creates a binding site for the SH2 domain of Grb2 (Schlaepfer & Hunter, 1996; Schlaepfer *et al.,* 1998). Thus, FAK appears to act as a scaffold for organizing a network of signaling and cytoskeletal proteins. Notably, FAK has been shown to be required for the integrin-initiated invasion processes induced by bacterial invasion factors,

including the invasin protein of *Y. pseudotuberculosis* (Alrutz & Isberg, 1998), the Fn-binding proteins of *S. aureus* (Agerer *et al.,* 2005) and invasive factors of *E. coli* (Reddy *et al.,* 2000). The role of FAK in *C. jejuni* invasion has not been investigated yet. Here, FAK-deficient cells and cells transfected with different FAK mutants were shown to be significantly impaired in their ability to internalize *C. jejuni*, demonstrating a requirement of FAK for *C. jejuni* uptake. Furthermore, an increased level of FAK phosphorylation at position Y397, indicating FAK activation, was observed after infection of FAK (+) cells with a wild-type isolate of *C. jejuni*. In contrast to this finding, no FAK phosphorylation was detected in cells inoculated with the *C. jejuni* F38011Δ*cadF* mutant. These results are consistent with a previous study, where an increase in the phosphorylation of paxillin, an event considered to be downstream of FAK activation (Richardson & Parsons, 1996), was observed upon cellular challenge with wild-type *C. jejuni* but not with the *C. jejuni cadF* mutant (Monteville *et al.,* 2003). In summary, my data suggest that CadF not only enables *C. jejuni* to bind to Fn for efficient attachment and invasion but appears to be involved in a signal transduction pathway leading to tyrosine phosphorylation of FAK and paxillin, which in turn can regulate host signaling events leading to actin rearrangement (Tachibana *et al*., 1995; Miyamoto *et al*., 1998). Accordingly, *C. jejuni*-triggered Rac1 and Cdc42 activation was abolished in the absence of FAK, thus demonstrating that FAK is essential in connecting an integrin initiated stimulus with Rho GTPases activation during *C. jejuni* invasion. This was further confirmed by my finding that F38011Δ*cadF* mutant less efficiently induced activation of Rho GTPases in FAK (+) cells compared to wt, result consistent with reduced activation of Rho GTPases in INT-407 cells infected with F38011Δ*cadF* mutant. However, the CadF mutant was still able to induce some residual GTPase activation suggesting that other bacterial factor(s) are also implicated in this signaling. The idea that the CadF is not the sole *C. jejuni* pathogenicity factor playing a role in signaling leading to the activation of Rac1 and Cdc42 was proved by the fact that no detectable activation was found in FAK (+) cells infected with flagellin mutant 81-176Δ*flaA/B* or flagellar biosynthesis mutant 81-176Δ*flhA* during the course of infection. Functional flagella have been previously shown to be required for *C. jejuni* invasion, but their involvement is as yet uncharacterized (Wassenaar *et al.,* 1991; Grant *et al.,* 1993; Yao *et al.,* 1994). Results presented here indicate significance of flagellar apparatus in activation of Rac1 and Cdc42 by *C. jejuni,* thus explaining a role of flagellar apparatus in *C. jejuni* uptake. However, whether flagella interact directly with host receptors or if flagellar apparatus serves as secretory apparatus for effector proteins remains to be elucidated in future studies. In summary, these findings suggest that both the CadF and flagella are able to trigger GTPase signaling in infected host cells.

6.6. Role of guanine nucleotide exchange factors in host cell invasion of *Campylobacter jejuni*

To determine signaling molecules downstream from integrins and FAK that could lead to Rac1 and Cdc42 activation during *C. jejuni* infection, the impact of a number of GEFs was analyzed. One candidate for a role in *C. jejuni*-mediated activation of Rho family GTPases is DOCK180 (Kiyokawa *et al.,* 1998). DOCK180 is an unconventional GEF for Rac1 devoid of the Dbl-homology/pleckstrin-homology tandem domains characteristic of Rho-family GEFs (Brugnera *et al.,* 2002; Cote & Vuori, 2002; Katoh & Negishi, 2003). DOCK180 forms a signaling complex with FAK, c-Src, $p130^{Cas}$ and Crk which in turn can activate Rac1 (Kiyokawa *et al.,* 1998; Gu *et al.,* 2001; Hsia *et al.,* 2003). DOCK180 appears to be a good candidate for linking Rac1 activation to *C. jejuni* invasion as down-regulation of DOCK180 using target-specific siRNA led to the significant reduction of *C. jejuni* uptake by host cells. This finding is consistent with reduced *C. jejuni* internalization observed by FAK (-) cells transfected with FAK Pro^- mutant lacking several proline residues necessary for association of SH3-containing $p130^{Cas}$ (Sieg *et al.,* 1999). In this context, it is interesting to note that DOCK180 has been recently implicated both in IpgB1-promoted *Shigella* entry (Handa *et al.,* 2007) as well as in InvA-mediated internalization of *Yersinia* (Bruce-Staskal *et al*., 2002; Wong & Isberg, 2005). Additionally, expression of Pro^- mutated FAK was reported to lead to a strong impairment of *S. aureus* uptake (Agerer *et al.,* 2005).

Another example of a GEF that can be activated downstream from integrin engagement is Vav-2 (Marcoux & Vuori, 2003). Vav-2 is a crucial downstream component in EGFR- and PI3-kinase-dependent Rac1 activation upon integrin-mediated cell adhesion (Marcoux & Vouri, 2003). It was reported to display GEF activity for Rac1, Cdc42, and RhoA (Abe *et al.,* 2000; Heo *et al.,* 2005). Notably, siRNA mediated gene silencing of Vav-2 and overexpression of several Vav-2 mutants significantly reduced the uptake of *C. jejuni* by host cells.

Other pathways downstream of integrins that eventually lead to GTPases activation may also be involved during *C. jejuni* infection. Paxillin is proposed to play a role in targeting effectors of activated Rac1 rather than stimulating Rac1 activation (Manser *et al.,* 1997; Brown *et al.,* 2002). For example, FAK can directly or indirectly interact *via* paxillin with the Cool/PIX family of proteins, GEFs for Rac1 and Cdc42 (Turner *et al.,* 1999; Zhao *et al.,* 2000). The existence of paxillin-paxillin kinase linker (PKL)-α-PIX-PAK-complex is required for recruitment of PAK to focal complexes and plays an important role in the regulation of Rac1 activity (Turner *et al*., 1999; West *et al*., 2001; Brown *et al.,* 2002). α-PIX can be activated by signaling cascades from the PDGFR and from integrin-induced signaling either by the formation of a complex with PAK and Nck, or direct association with the p85

regulatory subunit of PI3-kinase (Yoshii *et al.,* 1999). Reduction of *C. jejuni* invasion was observed after down-regulation of α-PIX with siRNA indicating a role of α-PIX in *C. jejuni* uptake by host cells. Interestingly, α-PIX has been also reported to interact with CagA protein during *H. pylori* infection of AGS cells which demonstrates that this GEF may be a common target of bacterial pathogens (Baek *et al.,* 2007).

Activated FAK can bind and phosphorylate a range of different substrates, which allow further recruitment of adaptor and signaling molecules. The phosphorylation of FAK at Y397 and subsequently at Y925 creates the SH2-binding site for the Grb2-SOS complex and provides a link to the activation of the Ras signal transduction pathway (Schlaepfer & Hunter, 1996). Ras can activate several downstream effectors including Rac1 (Bar-Sagi & Hall, 2000). Here, transfected FAK knockout fibroblasts re-expressing FAK Y397F or FAK Y925F mutants were impaired in *C. jejuni* invasion, suggesting that loss of Grb2 binding site reduces *C. jejuni* uptake. Interestingly, Lambert and co-workers (Lambert *et al.,* 2002) demonstrated that Tiam1, a Rac1-specific GEF, preferentially associates with activated GTP-bound Ras through a Ras-binding domain. Furthermore, activated Ras and Tiam1 cooperate to cause synergistic formation of Rac1-GTP in a PI-3-kinase-independent manner. Thus, it is tempting to speculate that Tiam1 could function as an effector that directly mediates Ras activation of Rac1 during *C. jejuni* invasion of host cells. In support of this notion both, down-regulation of Tiam1 and host cell treatment with Rac1 inhibitor, NSC23766 (Gao *et al.,* 2004) significantly reduced *C. jejuni* internalization by host cells. Although FAK and Trio comprise bi-directional signaling complex and Trio may be involved in effecting changes in the actin cytoskeleton trough the activation of Rho GTPases RhoA and Rac1 (Medley *et al.,* 2003), no role for Trio in *C. jejuni* invasion was found. Down-regulation of Trio had no effect on *C. jejuni* internalization.

The results of inhibitor studies suggest that Rac1 and Cdc42 are activated by at least two signaling pathways upon infection with *C. jejuni*. The activation of Cdc42 but not Rac1 was entirely blocked by tyrphostin-46 and wortmannin, suggesting that growth factor receptors and PI-3 kinase play a crucial role in the *C. jejuni*-induced activation of Cdc42. This indicates that Cdc42 is likely activated by a classical growth factor receptor (*e.g.* EGFR or PDGFR) and PI-3 kinase-dependent pathway. Importantly, both inhibitors had almost no effect on the activation of Rac1. This is remarkable since most of the known signaling pathways leading to the activation of Rac1 are PI-3 kinase dependent (Rottner *et al.,* 2004; Disanza *et al.,* 2005). The only known exception was shown for a PI-3 kinase-independent pathway involving Tiam1. Interestingly, the interaction between Tiam1 and the actin-related protein 2/3 (Arp2/3) complex links activation of Rac1 to actin polymerization (Ten Klooster *et al.,* 2006) and the intrinsic exchange factor activity specific for Rac1 is enhanced by threonine phosphorylation of Tiam1 (Michiels *et al.,* 1997; Fleming *et al.,* 1999; 2000).

In agreement with this hypothesis, it was observed that the activation of Rac1 is entirely blocked by staurosporine, a well-known inhibitor of threonine and serine kinases. Thus, one could hypothesize that *C. jejuni* CadF may activate Rac1 by a pathway involving serine and threonine kinases and Tiam1. The latter conclusions are consistent with previous inhibitor studies, demonstrating that *C. jejuni* entry into host target cells (as measured by gentamicin protection assays) is blocked by inhibitors including genistein, tyrphostin-46, staurosporine or wortmannin (Wooldridge *et al.,* 1996; Biswas *et al.,* 2000; 2004; Hu *et al.,* 2006a).

6.7. Impact of the epidermal growth factor and platelet-derived growth factor receptors for host cell invasion of *Campylobacter jejuni*

EGFR and PDGFR belong to RTK family of proteins, essential components of the signal transduction pathways that mediate cell-to-cell communication. These single-pass transmembrane receptors, which bind polypeptide ligands, mainly growth factors, play key roles in processes such as cellular growth, differentiation, metabolism and motility (Hubbard & Miller, 2007). Their role in host cell invasion of *C. jejuni* has not been investigated yet. Several lines of evidence support the hypothesis that Rac1 and Cdc42 can be activated downstream from EGFR/PDGFR through Vav-2 during *C. jejuni* infection. Vav-2 is a substrate of the EGFR and PDGFR (Liu & Burridge, 2000; Marcoux & Vuori, 2003; Tamas *et al.,* 2003), thus the first indications came form down regulation of Vav-2 and inhibitor studies as discussed above, followed by the findings with dominant-negative forms of EGFR/PDGFR and Vav-2. Inhibition of both receptors by tyrphostin-46 as well as transient transfection of host cells with both DN-EGFR/DN-PDGFR significantly reduced *C. jejuni* uptake. Furthermore, reduction in internalization of *C. jejuni* was observed in cells transfected with different Vav-2 mutants, findings consistent with reduced uptake of cells in which expression of Vav-2 was down regulated. Other studies have demonstrated that Vav-2 has the ability to reorganize the actin cytoskeleton through activation of Rho GTPases (Bustelo, 2000). Thus, Vav-2 could likely be a critical integrator of receptor signals and direct effectors of the changes in the actin cytoskeleton involved in *C. jejuni* invasion. Significantly, no additive reduction of *C. jejuni* uptake was noted upon transfection of host cells with both DN-EGFR and DN-PDGFR constructs. This finding is in a good agreement with results indicating that other signaling pathways are also implicated in *C. jejuni* internalization. Integrin occupancy, clustering and assembly of integrin-dependent signaling complexes can lead to ligand-independent phosphorylation and trans-activation of PDGFR and EGFR, followed by induction of downstream signaling (Miyamoto *et al.,* 1996; Moro *et al.,* 1998; 2002). Effects of this type have the potential to broaden the range of integrin signals and enhance their activity (Schwartz & Ginsberg, 2002). In this respect, it is tempting to speculate

that integrin and PDGFR/EGFR interact during *C. jejuni* infection leading to the stimulation of common downstream signaling pathways, including activation of FAK, Rac1 and Cdc42. This in turn causes actin rearrangements and efficient *C. jejuni* uptake.

6.8. Interplay between the microtubule and actin cytoskeleton during *Campylobacter jejuni* invasion

Microtubule-dependent host cell entry by *C. jejuni* is considered as one of the primary reasons for bacterial-caused tissue damage; however, the molecular mechanisms of *C. jejuni* invasion are widely unknown. Although interfering with microtubule organization using nocodazole has been shown to suppress *C. jejuni* uptake by host cells, the role of microtubules in *C. jejuni* invasion has not been fully clarified (Oelschlaeger *et al.,* 1993; Biswas *et al.,* 2000; 2003). Numerous reports suggest an actin-filament-dependent (microtubule-independent) and/or microtubule-dependent mechanisms by which *C. jejuni* invades gut tissue cells but no consensus has been established (Oelschlaeger *et al.,* 1993; Hu & Kopecko, 1999; Biswas *et al.,* 2000; 2003; Monteville *et al.,* 2003). Many studies are currently focused on unraveling the precise mechanism of cross-talk between the actin and microtubule systems. Recent observations indicate the co-ordination of signals between the actin and microtubule cytoskeleton, which is mediated through Rho family GTPases during cell locomotion (Waterman-Storer & Salmon, 1999; Wittmann & Waterman-Storer, 2001; Kaverina *et al.,* 2002). Rho GTPases can influence, or are influenced by, microtubule dynamics (Fukata *et al,* 2002; Ory *et al.,* 2002). Interestingly, Rho GTPases were reported to be regulated by the microtubule system during *Y. pseudotuberculosis* uptake (McGee *et al.,* 2003). The nocodazole effect on microtubule depolymerization was partially inhibited through overexpression of Rho GTPase family members and completely prevented by expression of their regulator Vav-2. This suggests that microtubules influence Rho GTPases during invasin-mediated internalization and in the absence of functional microtubules Vav-2 can mimic their effect on one, or more, of the Rho family GTPases. Moreover, it seems that an intact microtubule network, which implies a functional microtubule rail system, controls actin dynamics *via* Rho GTPases (McGee *et al.,* 2003). Hence, *C. jejuni* internalization could involve interplay between the microtubule and actin cytoskeleton, which is mediated by Rho GTPases. This remains to be investigated in future studies.

6.9. Downstream targets of Rac1 and Cdc42 possibly mediating *Campylobacter jejuni*-induced actin rearrangements

The identity of the downstream targets of Cdc42 and Rac1 that mediate *C. jejuni*-induced actin rearrangements is not known. A very good candidate is the Arp2/3 complex. This is a ubiquitous, eukaryotic actin-organizer capable of initiating actin nucleation, branching and cross-linking (Welch & Mullins, 2002). Notably, numerous pathogens, including *L. monocytogenes*, *Shigella*, *Salmonella* and EPEC employ host Arp2/3 complex to tailor actin remodeling (Cossart, 2000; Frischknecht & Way, 2001; Criss & Casanova, 2003). Activated Cdc42 and Rac1 can promote actin nucleation *via* Arp2/3 complex trough the family of Wiskott-Aldrich syndrome-proteins (WASP) and Scar/WAVE proteins, respectively (Symons *et al.,* 1996; Rohatgi *et al.,* 1999; Eden *et al.,* 2002; Millard *et al.,* 2004). In this study, the impact of a number of GEFs during *C. jejuni* infection was analyzed. GEFs not only activate Rho GTPases but also participate in the signaling to downstream effectors by either binding to these effectors directly or to scaffold proteins that complex with components of effector pathway (Zhou *et al.,* 1998; Wang *et al.,* 2004). For example, association of Tiam1 with the Arp2/3 complex has been recently shown to promote the local activation of Rac1, which is required for the subsequent activation of Arp2/3 complex proteins leading to actin filament assembly (Ten Kloster *et al.,* 2006). As several lines of evidence indicate involvement of Tiam1 in *C. jejuni*-mediated Rac1 activation and invasion, it is tempting to speculate that the Arp2/3 complex could mediate *C. jejuni*-induced actin rearrangements. Furthermore, in other systems, targets of both Rac1 and Cdc42 include PAK protein family (Gruenheid & Finlay, 2003; Cossart & Sansonetti, 2004; Rottner *et al.,* 2004). These serine/threonine kinases are engaged in multiple signaling pathways. Upon binding to either GTP-bound Rac1 or Cdc42, PAKs undergo autophosphorylation on multiple sites and become activated (Bagrodia & Cerione, 1999). PAKs may control the actin cytoskeleton through phosphorylation and subsequent activation of LIM kinase that promotes inactivation of the actin severing/depolymerizing activity of cofilin (Arber *et al.,* 1998; Edwards *et al*., 1999). In addition, PAKs function includes activation of the JNK/SAPK, p38 MAP kinase cascades (Bagrodia & Cerione, 1999; Hofmann *et al.,* 2004). Recent studies demonstrate that *C. jejuni* infection of host cells results in the activation of the Erk and p38 MAP kinase pathways (Watson & Galan, 2005; Hu *et al*., 2006a). Notably, in this study role of α-PIX in *C. jejuni* invasion has been shown. α-PIX is a PAK-binding partner that localizes at focal complexes with PAK (Manser *et al*. 1998). Interestingly, interaction of PAK with α-PIX leads to an increase in PAK activity and allows for the formation of GEF/GTPase/effector complex with a built-in positive feedback loop and mutual regulation of activity (Manser *et al.,* 1998; Obermeier *et al.,* 1998). All of these processes potentially stimulate localized rearrangements

of the actin cytoskeleton and *C. jejuni* uptake. However, how activated Rac1 and Cdc42 potentially regulate the downstream *C. jejuni* invasion pathway needs to be further elucidated in future studies.

6.10. A model of *Campylobacter jejuni* host cell invasion

In summary, results of this study develop detailed understanding of unique mechanism by which *C. jejuni* invade host target cells. The current hypothetical model of *C. jejuni* host cell entry presented in Figure 42 is based on results discussed above and implicates Rac1 and Cdc42 as main players mediating *C. jejuni* uptake in epithelial cells. In this scenario, bacteria bound to Fn *via* CadF trigger Rac1 and Cdc42 activation through interactions with integrin receptors, resulting in actin cytoskeleton rearrangements that lead to bacterial internalization.

6.11. Role of the surface array protein SapA in infection of host cells with *Campylobacter fetus*

Another important finding of my work is that I established a role of SapA and SapA phosphorylation in *C. fetus* invasion. The presented data provide a first detailed study of SapA tyrosine phosphorylation and its role in *C. fetus* pathogenesis. Significantly, Src kinase phosphorylated purified SapA protein *in vitro* as well as SapA transiently expressed in cultured cells *in vivo.* Furthermore, the Src-specific tyrosine kinase inhibitor PP2 specifically blocked SapA phosphorylation in *C. fetus* infected cells and reduced invasion of SapA-expressing strains, but had no effect on low invasion rates of SapA-non-expressing strains. This clearly demonstrates that Src-like PTKs mediate SapA phosphorylation and indicates an important role of SapA phosphorylation during *C. fetus* infection.

The tyrosine phosphorylation of proteins has a central role during signal transduction in eukaryotes and many signaling cascades involve this highly regulated post-translational modification (Blume-Jensen & Hunter, 2001; Pawson, 2004). Interestingly, tyrosine phosphorylation of injected bacterial proteins is an emerging cellular signaling mechanism among pathogenic micro-organisms (Backert & Selbach, 2005). Directed injection of bacterial virulence factors into host target cells has been described for two distinct secretion machineries, namely T3SS and T4SS. After translocation, these effector molecules target various components of eukaryotic signaling pathways in order to mediate bacterial attachment or entry, to transform the host cell or to block bacterial uptake by phagocytosis (Hueck, 1998; Christie & Vogel, 2000; Lee & Schneewind, 2001).

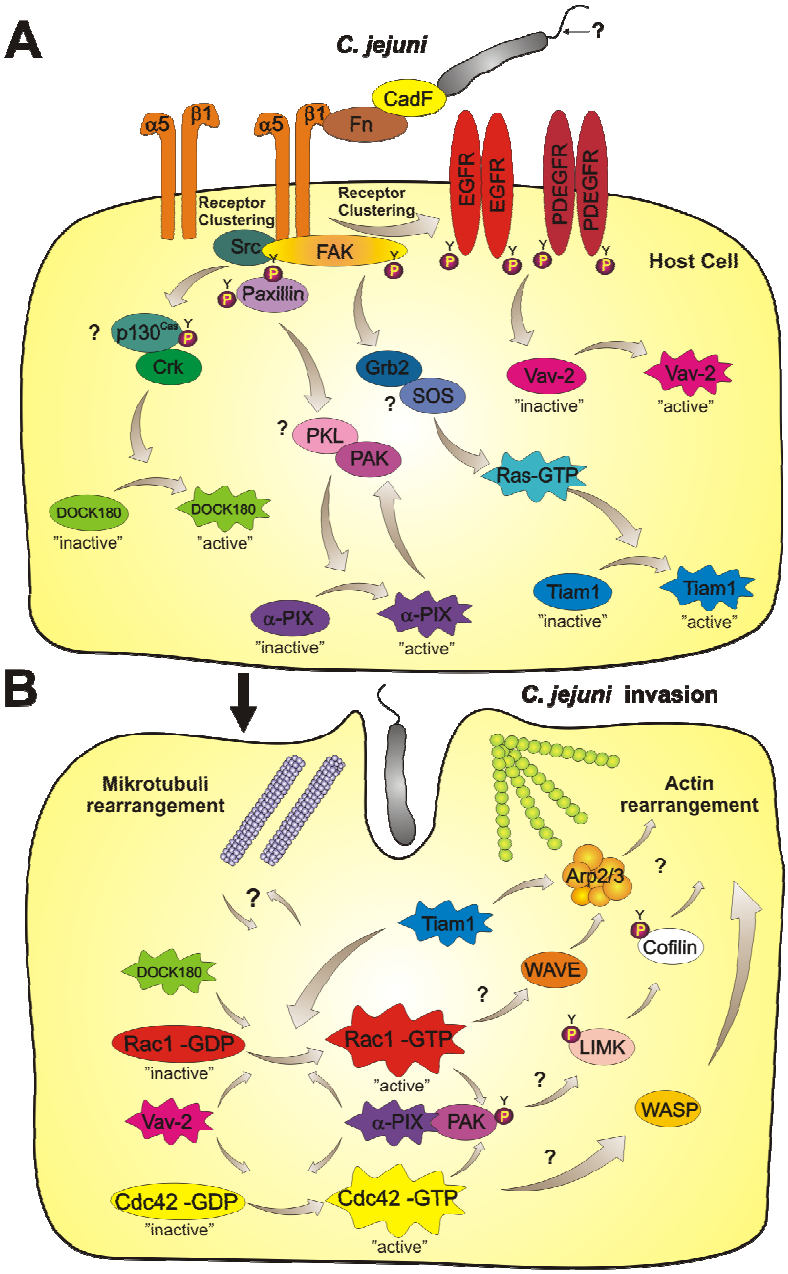


Fig. 42. Hypothetical model of *C. jejuni* invasion. (A) *C. jejuni* adheres to host cells *via* the Fn binding protein CadF which acts as a bridge engaging integrin receptors. Integrin occupancy and clustering leads to recruitment and activation of FAK. Active FAK and Src phosphorylate paxillin and $p130^{CAS}$. Phosphorylation of FAK creates binding site for Grb2. These events in turn trigger a cascade of signals resulting in formation of signaling complexes leading to activation of GEFs such as DOCK180, α-PIX and Tiam1. Assembly of integrin-dependent complexes leads to ligand-independent phosphorylation and trans-activation of PDGFR and EGFR, followed by stimulation of Vav-2. (B) Activated DOCK180, Tiam1, α-PIX and Vav-2 induce the exchange of GDP for GTP to generate the active forms of Rac1 and Cdc42. Upon binding to GTP-bound Rac1 or Cdc42, PAK becomes activated and phosphorylates LIM kinase that promotes inactivation of the actin depolymerizing activity of cofilin. Whereas interaction of PAK with α-PIX leads to an increase in PAK activity, association of Tiam1 with the Arp2/3 complex promote activation of Rac1 and provide a link to actin polymerization. All of these signaling pathways potentially cause localized actin rearrangements at the site of *C. jejuni* entry, resulting in bacterial uptake. Co-ordination of signals between the actin and microtubule cytoskeleton, mediated by Rho family GTPases during *C. jejuni* invasion needs to be elucidated in future studies.

A good example is the translocated intimin receptor (Tir) of EPEC that, after injection and tyrosine phosphorylation, serves as a receptor for intimin adhesin (Kenny *et al.,* 1997; Deibel *et al.,* 1998). Furthermore, by recruiting Nck, phosphorylated Tir can initiate actin pedestal formation in host cells (Gruenheid *et al.,* 2001). Similar to EPEC, the mouse pathogen *Citrobacter rodentium* expresses Tir, and phosphorylated Tir is essential for actin polymerization (Deng *et al.,* 2003). Recently, effector proteins of *Chlamydia trachomatis* and *Bartonella henselae*, the translocated actin-recruiting phosphoprotein (Tarp) and *Bartonella*-effector protein D (BepD), respectively, were shown to be phosphorylated on tyrosine residues after translocation into host cells (Clifton *et al.,* 2004; Schulein *et al.,* 2005). Whereas phosphorylated Tarp may stimulate actin-driven *C. trachomatis* invasion, the cellular effects of phosphorylated BepD remain to be defined (Clifton *et al.,* 2004; Schulein *et al.,* 2005). *H. pylori* CagA is another example of a bacterial effector protein that upon translocation into the host cells undergoes tyrosine phosphorylation, mediated by two PTKs Src and Abl (Segal *et al*., 1999; Backert *et al*., 2000; Odenbreit *et al*., 2000; Selbach *et al.,* 2002; Tammer *et al.,* 2007). CagA phosphorylation induces rearrangements of the host cell actin cytoskeleton and cell scattering (Backert *et al*., 2001; Stein *et al*., 2002) and is required for binding to the Shp-2 tyrosine phosphatase (Higashi *et al*., 2002), the Csk tyrosine kinase (Tsutsumi *et al*., 2003) and FAK (Tsutsumi *et al*., 2006). Interestingly, all named bacterial proteins are effector proteins, which upon translocation into the host cells undergo tyrosine phosphorylation in order to target various components of eukaryotic signaling pathways. These injected molecules appear as host proteins but act as Trojan horses containing a bacterial hidden core message that allows the microorganism to take control over the host cell. Although the exact role of phosphorylated SapA during *C. fetus* invasion remains to be investigated, it seems that SapA is the first non-effector bacterial protein, which becomes tyrosine-phosphorylated by the host cell kinases. All named pathogens use the T3SS or T4SS to inject their effector proteins into host cells, in contrast, SapA belongs to the surface array proteins forming S-layer of *C. fetus* and T1SS has been shown to be responsible for its transport to the bacterial surface (Thompson *et al.,* 1998). S-layers, or paracrystalline surface protein arrays, constitute the outermost component of several gram-negative and gram-positive bacteria (Bahl *et al.,* 1997; Sara & Sleytr, 2000). Diverse functions have been proposed for S-layers, such as acting as protective coats, cell shape determinants, and promoters for cell adhesion and surface recognition; however, a general function for all S-layers has not been determined (Beveridge *et al.,* 1997; Sara & Sleytr, 2000). There is increasing evidence that S-layers can contribute to virulence when they are present as a structural component of the cell envelope of pathogens. For example, the S-layer of *Aeromonas salmonicida* contributes to protection against the bactericidal activities of both nonimmune and immune sera. Additionally, *Aeromonas* mutants unable to produce an

S-layer are altered in their ability to cause disease (Noonan & Trust, 1997). A similar observation was reported for the S-layer from *Bacillus cereus* isolated from periodontal infections. Only the S-layer-carrying cells were resistant to polymorphonuclear leukocytes in the absence of opsonins (Kotiranta *et al.,* 1998). The *Campylobacter rectus* S-layer confers resistance to complement-mediated killing and causes the down-regulation of proinflammatory cytokines (Thompson, 2002). Evidence from experiments using bovine and human *C. fetus* isolates suggests that S-layer is the predominant virulence factor for this organism (Pei & Blaser, 1990). *C. fetus* wt strains, possessing S-layer, are highly resistant to the bactericidal activity of normal serum and ingestion by phagocytes, which correlates with their ability to cause bacteraemia (Blaser *et al.,* 1988). In contrast, mutant strains lacking the S-layer, are sensitive to both serum- and phagocytosis-mediated killing and have reduced virulence in both ungulate (Grogono-Thomas *et al.,* 1996; 2000) and rodent models (Blaser & Pei, 1993). These findings are in a good agreement with results presented in this study. Namely, *C. fetus* strains expressing SapA exhibited significantly higher invasion rates and showed enhanced ability to trigger IL-8 release from host cells than SapA-non-expressing mutants lacking S-layer. This indicates a role of SapA in *C. fetus* invasion and adds a totally new aspect to *C. fetus* pathogenesis. Intracellular location can protect *C. fetus* from host innate and adaptive immune defenses and establish a reservoir of infecting bacteria, thereby contributing to persistence of this organism within a host.

Interestingly, in recent years, the involvement of the S-layer in bacterial adhesion and in bacteria-host interactions has attracted interest due to the potential association with bacterial virulence. S-layers have been shown to function as adhesins in several pathogenic and commensal bacteria, mediating binding to epithelial cells and/or ECM (Kotiranta *et al.,* 1998; Hynonen *et al.,* 2002). Attachment to laminin and Fn in *Aeromonas salmonicida* (Doing *et al.,* 1992; Noonan & Trust, 1997), to avian intestinal epithelial cells in *Lactobacillus acidophilus* (Schneitz *et al.,* 1993), and to collagen in *Lactobacillus crispatus* (Toba *et al.,* 1995) has been reported to require S-layer. Recently, S-layer of *Lactobacillus brevis* has been demonstrated to function as an adhesin to human epithelial cells and Fn, laminin, fibrinogen as well as collagen (Hynonen *et al.,* 2002; de Leeuw *et al.,* 2006). Furthermore, binding of *Bacillus cereus* to laminin is mediated by the S-layer (Kotiranta, 1998; 2000). However, here it was shown that despite a prominent cell surface localization, SapA does not function as a primary adhesin for *C. fetus*. SapA-non expressing *C. fetus* strains adhered to host cells at equivalent levels as wt strains and no correlation between SapA expression and *C. fetus* adherence was observed, confirming results of previous study (Graham & MacDonald, 1998). Furthermore, host cells did not bind to SapA-coated surfaces. It seems that unlike the S-layer of *A. salmonicida*, which functions as an adhesin that promotes but

does not mediate invasion (Garduno *et al.,* 2000), SapA can contribute to the virulence of *C. fetus* by enhancing its internalization into host cells.

It is becoming clear that *C. fetus* has evolved many mechanisms for survival in an immunologically hostile host and enabling chronic infection. It appears that S-layer not only gives *C. fetus* protection from the alternative pathway of complement and permits the evasion of antibodies *via* antigenic variation but also in phosphorylated form plays a role in *C. fetus* internalization of host cells. However, it remains a challenge to identify the host proteins that interact with phosphorylated SapA and to decipher its exact role in invasion and pathogenesis during *C. fetus* infection.

6.12. Conclusions

The interaction of *C. jejuni* with epithelial cells has the clinical consequences of an inflammatory response and enteritis. Therefore, detailed understanding of the signaling events triggered by *C. jejuni* infection, presented here, should help to explain its nature and may lead to the development of novel therapeutic strategies to limit the clinical consequences of inflammatory diarrhea. Findings of this study elucidating *C. jejuni* invasion mechanism, not only reveal important molecular details of the cell biology of this process, but also add important aspects to the understanding of *Campylobacter*-induced pathogenesis.

Taken together, the work presented here identifies and characterizes CadF protein as important pathogenicity factor expressed in all tested *C. jejuni* and *C. coli* strains and involved not only in adhesion but also required for maximal invasion of *Campylobacter*. Additionally, the data strongly suggest that the differences in molecular size and nucleotide sequence between CadF of *C. jejuni* and *C. coli* isolates may be a suitable diagnostic marker to discriminate between these species in food and clinical specimen. Moreover, CadF is proposed to be a bi-functional protein, acting as both a canonical adhesin for bacterial binding to Fn and also stimulating integrin clustering, which subsequently can activate downstream factors triggering GTPase signaling in infected host cells.

Furthermore, this study demonstrates several lines of evidence for a role of Rac1 and Cdc42 but not RhoA during host cell entry of *C. jejuni*. Additionally, integrins, EGFR, PDGFR, FAK, DOCK180, Vav-2, α-PIX and Tiam1 are critically involved in mediating *C. jejuni* invasion-promoting signals. In this scenario activated integrins and PDGFR/EGFR interact during *C. jejuni* infection and trigger formation of various signaling complexes including FAK, DOCK180, Vav-2, α-PIX and Tiam1 leading to the activation of Rac1 and Cdc42 and stimulation of common downstream signaling pathways. This in turn causes actin rearrangement and efficient *C. jejuni* uptake, as summarized in Figure 42.

Finally, the presented data provide a first detailed study of SapA tyrosine phosphorylation and its importance in *C. fetus* pathogenesis, clearly showing that Src-like PTKs mediate SapA phosphorylation and indicate significant role of phosphorylated SapA during infection.

There remain many open areas of investigation regarding the analysis of signal transduction events during *C. jejuni* invasion. It is worth pointing out that although here Rac1 and Cdc42 have been placed as the central players transmitting signals to the actin cytoskeleton after bacterial engagement of integrin receptors, there are many intermediary factors upstream and downstream of Rac1 and Cdc42 linking activated integrins to the actin cytoskeleton, which have not been studied in detail here. Thus, further work can provide more information about how activated Rac1 and Cdc42 potentially regulate the downstream *C. jejuni* invasion pathway and decipher the importance of the different signal transduction events that potentially lead to Rac1 and Cdc42 activation during *C. jejuni* uptake. Clearly, additional studies on interplay between the microtubule and actin cytoskeleton, potentially mediated by Rho GTPases during *C. jejuni* invasion, would be highly interesting. Finally, defining the precise mechanisms of how *C. jejuni* flagellar apparatus induces activation of Rac1 and Cdc42 to regulate actin rearrangement and microtubule dynamics involved in the bacterial entry process will be of particular interest. Last but not least, the exact function of tyrosine phosphorylated SapA during *C. fetus* infection remains to be elucidated in future studies.

Undoubtedly, the expanded knowledge of the molecular mechanisms of *C. jejuni* and *C. fetus* pathogenesis will lead to improved methods for chemotherapeutic and prophylactic intervention of diarrhea, enteritis and bacteraemia.

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8. Erklärung

Hiermit erkläre ich, dass ich die von mir eingereichte Dissertation zum Thema „Pathogenicity mechanisms of *Campylobacter jejuni* and *Campylobacter fetus*: characterization of pathogenicity factors and signaling in host cell invasion“ selbstständig verfasst, nicht schon als Dissertation verwendet habe und die benutzten Hilfsmittel und Quellen vollständig angegeben wurden.

Weiterhin erkläre ich, dass ich weder diese noch eine andere Arbeit zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.) an anderen Einrichtungen eingereicht habe.

Magdeburg, den 12.11.2007

Małgorzata Krause-Gruszczyńska

9. Curriculum Vitae

Personal information

Name	Małgorzata Anna Krause-Gruszczyńska
Date of birth	05.10.1979
Place of birth	Gdynia
Nationality	polish
Address	Am Heiderand 26 06120 Halle

Education

1994 - 1998	Secondary School Nr. 3 Gdansk, Poland
1998 - 2003	Biotechnology Studies at the Intercollegiate Faculty of Biotechnology, Medical University of Gdansk and University of Gdansk, Poland The experimental work towards Master thesis: „The role of pyruvate kinase in *Corynebacterium glutamicum*." was carried out at the Institute of Biotechnology 1, Forschungszentrum Jülich, Jülich, Germany.
Degree:	Master of Science in Biotechnology

Previous laboratory experience

October 2000 - March 2001	Institute of Forensic Medicine, Medical University of Gdansk, Poland
October 2001 - February 2003	Institute of Biotechnology 1, Forschungszentrum Jülich, Jülich, Germany

Scholarships and Awards

October 2001-August 2002,	SOCRATES/ ERASMUS Scholarship
October 2002	Award from President of City Gdansk for very good results in academic year 2001/2002
Juli 2005-Juni 2006, September 2006-Februar 2007	PhD Scholarship from Magdeburger Forschungsverbund

PhD

From February 2004	Institute of Medical Microbiology, Otto-von-Guericke-University Magdeburg PhD Thesis: „Pathogenicity mechanisms of *Campylobacter jejuni* and *Campylobacter fetus*: characterization of pathogenicity factors and signaling in host cell invasion"

Magdeburg, 12.11.2007

Małgorzata Krause-Gruszczyńska